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Regulation of glyoxysomal enzyme gene expression in loblolly pine (*Pinus taeda* L.) seeds following imbibition

by

Robert Thomas Mullen



A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

in

Plant Molecular Biology

Department of Botany

Edmonton, Alberta

Fall, 1995



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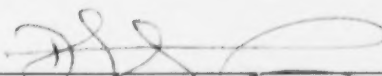
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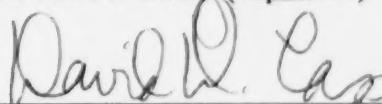
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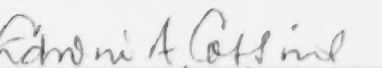
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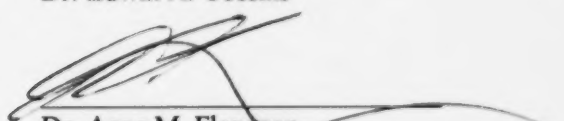
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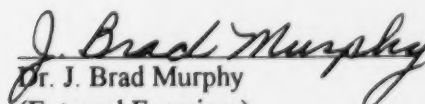


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To Judy, Stephanie and Erin

For all your patience, understanding and love

ABSTRACT

Following the completion of germination, oil seeds such as loblolly pine (*Pinus taeda* L.) begin to mobilize lipid reserves via the β -oxidation of fatty acids. The subsequent conversion of acetyl-CoA to malate (via the glyoxylate cycle) provides the substrate for carbohydrates that will go on to serve as the primary energy source of the developing young seedling. Although many of the enzymes involved in early seedling metabolism have been well characterized in angiosperms, any understanding of their developmental regulation in gymnosperms is limited. To begin to examine the developmental controls of gene expression following seed imbibition, and provide some much-needed basic information concerning these processes in conifers, the enzymes catalase (CAT), isocitrate lyase (ICL), and malate synthase (MS) were chosen as markers for study. CAT, ICL, and MS are key glyoxysomal enzymes, whose activities display a concerted and dramatic increase following seed germination. CAT, ICL, and MS were purified to homogeneity from germinated loblolly pine megagametophytes and monospecific antibodies for each enzyme elicited in rabbits. Next, a λ ZAP cDNA library was constructed from poly(A)⁺ RNA isolated from germinated megagametophytes and used for the isolation of CAT and ICL cDNA clones. In total, two full-length cDNAs encoding isoenzymes of ICL and a cDNA encoding the 5' half of the CAT protein coding region were isolated and sequenced to entirety. A comparison of the deduced amino acid sequences of loblolly pine CAT and ICL cDNAs to their angiosperm counterparts revealed a high degree of identity. With monospecific antibodies and cDNA clones as molecular

tools, the temporal, concerted development of CAT, ICL, and MS enzyme activities were examined in more detail. Protein and RNA blot analysis indicated that CAT and ICL are regulated primarily at the transcriptional level; CAT and ICL transcripts accumulated approximately 1-2 d before peak protein and enzyme levels. However, an examination of the rates of CAT and ICL *de novo* protein synthesis indicated that translational or post-translational processes also differentially affected the accumulation of protein.

A comparison of CAT, ICL, and MS synthesized *in vitro* and *in vivo* indicated that all three enzymes were synthesized at their final size, and therefore the targeting information for these proteins to the glyoxysome must reside in their mature polypeptides. To date, studies of other plant glyoxysomal/peroxisomal enzymes have identified a C-terminal peroxisomal targeting signal typified by the tripeptide serine-lysine-leucine (SKL). An analysis of the deduced amino acid sequence of the cDNAs for loblolly pine ICL revealed that the C-terminus for both proteins contain a conserved variant of the SKL motif. Results from these studies, and immunoblot experiments with antibodies raised to an SKL peptide, suggest that this topogenic signal may be used in the protein translocation of loblolly pine ICL and CAT, and that this targeting characteristic is likely a conserved mechanism between gymnosperms and angiosperms.

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp; kb	base pair; kilobase
bromophenol blue	3', 3', 5', 5'-tetrabromophenol-sulphonphthalein
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary DNA
Ci; mCi; μ Ci	Curie; milliCurie; microCurie
CoA	coenzyme A
Con A	concanavalin A
Da; kDa	dalton, kilodalton
DAB	3,3'-diaminobenzidine
DAI	days after imbibition at 30°C
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dNTP	2'-deoxynucleotide 5'-triphosphate
DE-52	diethylaminoethyl
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DOC	days of chilling at 2°C
dpm	disintegrations per minute
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
Endoglycosidase F	endo- β -N-Acetylglucosaminidase F:

FPLC	Endoglycosidase F
FW	fast pressure liquid chromatography
hr; min; sec	fresh weight
HTP	hour; minute; second
ICL	hydroxylapatite
IEF	isocitrate lyase
IgG	isoelectric focusing
IPTG	immunoglobulin G
<i>lacZ</i>	isopropyl β -D-Thiogalactoside
LEA	β -galactosidase gene
MS	late embryogenesis abundant
MOPS	malate synthase
mRNA	3-(N-Morpholino)propanesulphonic acid
NBT	messenger ribonucleic acid
PAGE	nitroblue tetrazolium
PBS	polyacrylamide gel electrophoresis
PCR	phosphate buffered saline
PEG	polymerase chain reaction
pI	polyethylene glycol
PMSF	isoelectric point
PNGase F	phenylmethylsulphonylfluoride
PTS	Peptide: N-glycosidase F
PVP	peroxisomal targeting signal
RNA	polyvinylpyrrolidone
RNase A; RNase B	ribonucleic acid
poly(A) ⁺ RNA	ribonuclease A; ribonuclease B
rpm	polyadenylated ribonucleic acid
<i>S.aureus</i>	revolutions per minute
SDS	<i>Stapylococcus aureus</i>
	sodium dodecyl sulphate

SKL	serine-lysine-leucine
<i>Taq</i>	<i>Thermus aquaticus</i>
TBS	Tris buffered saline
TCA	trichloroacetic acid
TE	10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
Tris	tris (hydroxymethyl) aminomethane
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

1. INTRODUCTION

1.1. Loblolly pine

Economically, conifers are important tree species for the production of pulpwood and lumber. With a number of attributes favored by the forest industry, including its acceptable wood properties and tremendous genetic variability, loblolly pine (*Pinus taeda* L.) has come to represent the single most important timber species in the southeastern United States (Zobel, 1982). As of 1992, over 80% of all pines planted in the southern pine regions were loblolly (Skinner, 1992). Loblolly pine or "oldfield pine" occurs naturally in the coastal plain and piedmont from Delaware to central Florida, eastern Texas, southern Arkansas, and southern Tennessee (Fowells, 1965) (Figure 1.1).

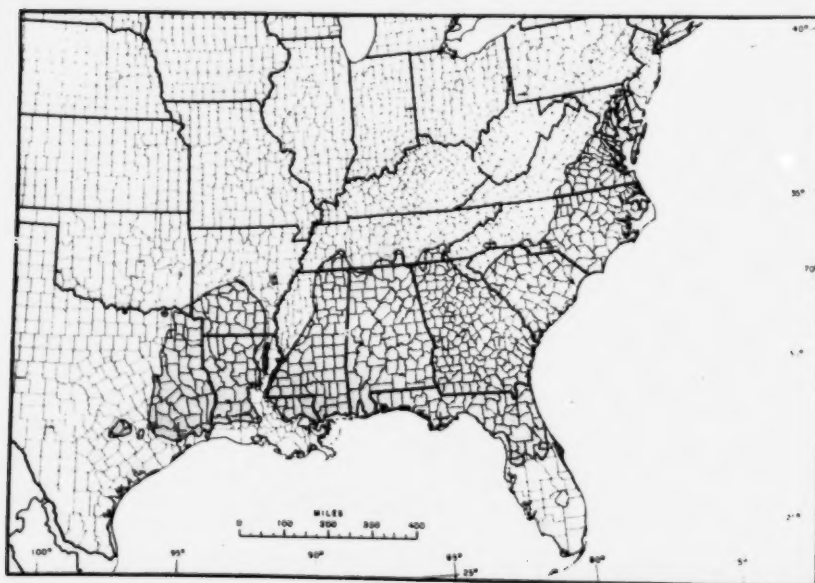


FIGURE 1.1. Map of the natural distribution of loblolly pine. Taken from Zobel (1982).

With the exception of the poplars, loblolly pine is the most intensively studied forest tree species in the world. The primary areas of study of loblolly pine have centered on tree improvement and changes to the general wood quality of the species (Kellison and Gingrich, 1982). However, as reforestation and afforestation practices attempt to compensate for current increasing demand for wood, pulp and paper products, more effort is being expended in developing a dependable supply of high quality seed. To this end, most of the supplies of loblolly pine seed are obtained from cultivated seed orchards, established by planting clones (grafts) or seedling progeny of known select trees (Shepard, 1986). Although these practices are successful in ensuring the preferred genetic qualities of the tree are maintained, subsequent storage and seed germination represent a major problem in nearly all economically important conifers species, including loblolly pine. Not only do the seeds require a long period of chilling or stratification, but germination rates and seedling vigor are generally poor (Edwards, 1986).

1.2. Germination

Following the period of quiescence imposed during late embryogenesis, seed germination starts with the imbibition of water and results in the rapid recommencement of metabolic processes including respiration and protein and RNA synthesis. Subsequently, the growing radicle emerges from the seed, marking the completion of germination, and seedling growth ensues (Bewley and Black, 1994). In total, these developmental transitions represent the most significant stage in the life cycle of higher plants. As a result, and in an integrated attempt to understand the biochemical and molecular events which accompany seed germination and early seedling growth, these developmental processes have been extensively studied in angiosperms (see Bewley and Marcus, 1990; Thomas, 1993; Bewley and Black, 1994 for reviews). In this review, I will focus primarily on studies that have examined germination and post-germination events in conifers. From this viewpoint, I will concentrate on the aspects of gene expression during germination, seed storage reserve mobilization, and report on the progress-to-date in lipid catabolism in conifer seeds.

1.2.1. Differential Gene Expression Underlies Conifer Seed Germination

Seed imbibition results in the rapid increase in the rates of respiration and protein and RNA synthesis. In terms of relative timing, in angiosperms it has been unequivocally demonstrated that polyribosome formation can commence before RNA synthesis. Here stored mRNAs, preformed during development, are available immediately on hydration and can be quickly translated into proteins that are essential for germination (reviewed by Payne, 1976). In a similar fashion, the translational components, as well as mRNA preformed during the later stages of seed maturation, persist in the mature conifer seed (Yamamoto, 1982; Whitmore, 1991). In *Pinus thunbergii*, Masumori et al. (1992) quantified the changes in several stored RNA species following germination. Using RNA blot hybridization with cDNA clones of stored mRNA species, Masumori et al. (1992) demonstrated that one of the mRNAs disappeared rapidly during germination, while others were detected at the later stages of germination. All the stored mRNA species examined were preserved in seeds that had been stored up to 14 years, suggesting that the mRNA was present in the mature seed as some stabilized complex. Although the relative functional significance of these mRNA species was not determined, Masumori et al. (1992) concluded that stored mRNA in mature pine seeds are composed of several subsets and are likely stored as ribonucleoprotein complexes.

At the onset of germination and through to post-germinative growth, unique patterns of gene expression have been identified that correspond with the distinct physiological changes occurring in the conifer seed during these periods. For example, in lodgepole pine (Gifford et al., 1991) and loblolly pine (Groome et al., 1991), electrophoretic analysis of proteins synthesized *in vivo*, indicated that unique protein sets were synthesized in a temporal fashion. Gifford et al. (1991) categorized these proteins into three groups; a first set synthesized constitutively throughout germination and post-germinative growth, a second set present in the mature seed and only synthesized during

early germination, and finally, a third set whose initial synthesis coincided with the completion of germination, and increased thereafter. In Norway spruce, Stabel et al., (1990) reported a similar differential synthesis of protein sets during germination and post-germinative growth. Although a biochemical role for these proteins or protein subsets was not assigned, Gifford et al. (1991) proposed that the third subset, or the group of proteins synthesized at high rates during post-germinative growth likely represent hydrolytic enzymes involved in the mobilization of seed storage reserves. In total, the results from these studies have indicated that the synthesis of distinct groups of proteins during conifer germination and post-germinative growth is likely the result of the expression of developmentally regulated genes.

In relation to this, Sundås et al. (1992) recently demonstrated that the *de novo* protein synthesis during Norway spruce embryo germination and post-germination reported earlier (Stabel et al., 1990) was correlated with the expression of specific cDNAs. In Norway spruce embryos, cytokinin treatment suppressed germinative development and induced the coordinate formation of adventitious buds. When analyzing the patterns of gene expression associated with germination and the alterations induced by cytokinin bud induction, Sundås et al. (1992) isolated a total of 14 cDNA clones which hybridized to transcripts that were abundant specifically during germination. Sundås et al. (1992) also isolated a second category of 13 clones that hybridized to transcripts that increased in abundance during post-germinative development of the seedling. Further analysis of the proteins encoded by these cDNAs identified a number of functional proteins including a replication dependent histone H2A (Sundås et al. 1993), an α -tubulin, the large subunit of ribulose biphosphate/carboxylase oxygenase, two subunits of a chloroplast ATPase, and a protein with a partial identity to a barley (1 $\rightarrow$ 3)- β -glucanase protein. In total, Sundås et al. (1992) concluded that a majority of the differentially expressed cDNAs isolated appeared to encode proteins that were involved in processes related to either cell division in the germinating hypocotyl, or greening of the cotyledons during post-germinative growth.

More recently, and to further characterize the general patterns of gene expression during conifer germination and post-germinative growth, Tranbarger and Misra (1994)

isolated and characterized a set of cDNAs from a Douglas-fir germination-specific library. In total, five partial cDNA clones were isolated with homology to a range of plant proteins including; a chaperonin 60 β , a low-molecular-weight heat shock protein, a luminal binding protein, a type II chlorophyll a/b-binding protein, and a cysteine protease. Northern blot analysis indicated that each cDNA hybridized to mRNAs that accumulated in a differential fashion during and following seed germination in Douglas-fir. Tranbarger and Misra (1994) concluded that each of these cDNAs encoded proteins that possessed some biochemical role during the physiological changes that occur in the megagametophyte and embryo of germinating conifer seeds.

1.2.2. Seed Storage Reserves

In angiosperms, the later stages of seed maturation represent a unique developmental period characterized by cell expansion and deposition of reserves (normally proteins, along with lipids or carbohydrates) in the storage tissues (i.e. endosperm or cotyledons). In general, studies of angiosperms have shown that the regulation of the expression of genes involved in seed storage reserve synthesis is primarily transcriptional (Quatrano, 1986; Goldberg et al., 1989; Lopes and Larkins, 1993; Murphy, 1993; West and Harada, 1993). In conifer seeds, the majority of the stored seed reserves are lipids and proteins and are localized within the haploid megagametophyte (Figure 1.2) (Ching, 1966; Baron, 1970; Salmia, 1981; Cardemil and Reinerio, 1982; Kovac and Wrischer, 1984; Gifford, 1988; Gifford and Tolley, 1989; Kovac and Kregar, 1989; Misra and Green, 1990; Flinn et al., 1991a; Groome et al., 1991). Following germination, these reserves are hydrolyzed, and the liberated free amino acids and sugars are transported to the developing seedling and used as a primary nitrogen or energy source, respectively, until photoautotrophic independence is attained (Ching, 1972; Higgins, 1984; Lammer and Gifford, 1989).

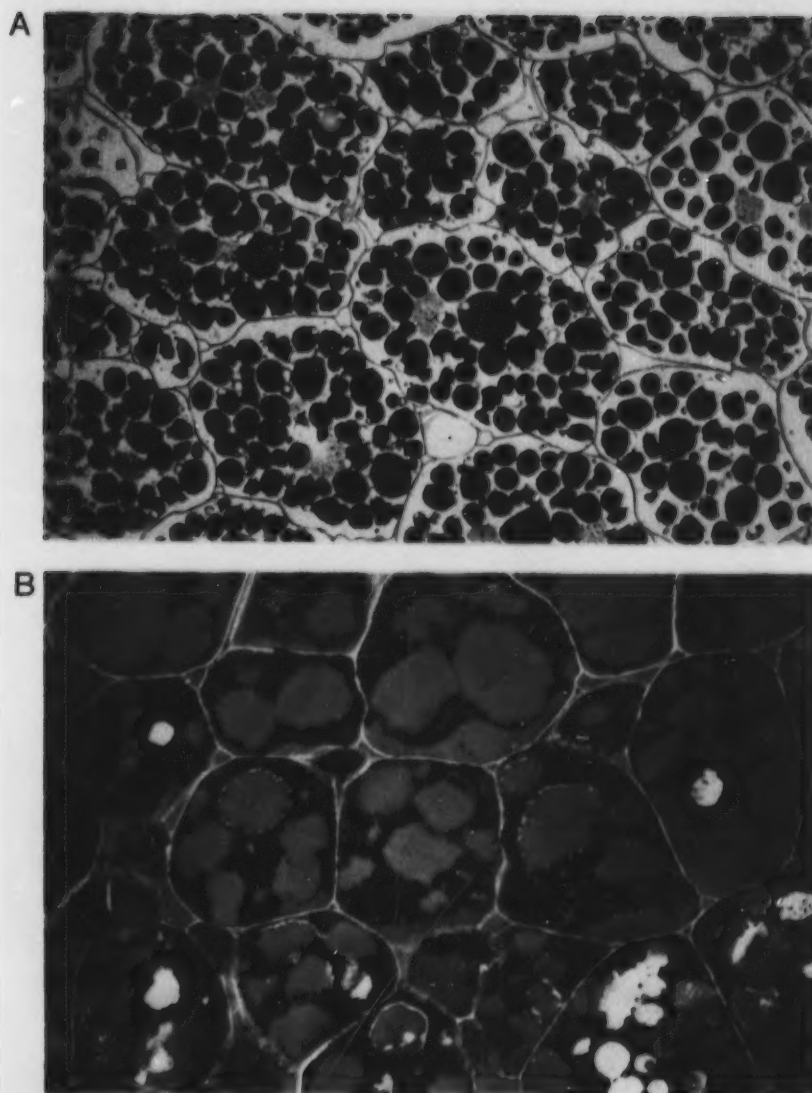


FIGURE 1.2. Light micrograph of protein and lipid storage reserves in the megagametophyte of mature loblolly pine seeds. A mature loblolly pine megagametophyte was fixed, section and stained for proteins (panel A) or lipids (panel B). Photo provided by Stone and Gifford (unpublished)

Traditionally, storage proteins have been classified according to their solubilities; the albumins are soluble in water, globulins are soluble in a salt solution, glutelins in dilute acids or alkaline solution, and the prolamins in aqueous alcohol (Higgins, 1984; Bewley and Black, 1994). In angiosperms, extensive research has led to a detailed understanding of the types, synthesis, processing, and molecular regulation of genes encoding seed storage protein in a variety of seed types (see Shotwell and Larkins, 1989 for a review). In general, seed storage proteins are encoded by complex gene families, synthesized on the rough endoplasmic reticulum, and typically undergo a number of post-translational modifications including signal sequence cleavage, glycosylation, oligomeric association, and combinations of intermolecular and intramolecular disulfide bridge formation. Once processed, the proteins are deposited, via the Golgi apparatus, to membrane-bound organelles called protein bodies (Boulter, 1981; Shotwell and Larkins, 1989; Bewley and Black, 1994).

Although far less extensively examined than in angiosperms, a number of studies have begun to qualify the types of conifer seed storage proteins, as well as the regulation of their synthesis. Characterized by their solubility in buffer solutions containing SDS or urea, the majority of storage proteins in many conifer species are crystalloids (Gifford, 1988; Gifford and Tolley, 1989; Jensen and Berthold, 1989; Lammer and Gifford, 1989; Misra and Green, 1990, 1991; Roberts et al., 1990; Groome et al., 1991; Hackman, 1993). In mature seeds of loblolly pine, the crystalloid proteins account for over 85% of the buffer-insoluble proteins (Groome et al., 1991). Similarly in Douglas-fir, Green et al. (1990) reported that these proteins account for over 75% of the storage proteins, and demonstrated their localization in protein bodies with electron microscopy. In castor bean, the quaternary structure of the crystalloid protein is characterized by a holoprotein of 300 kDa comprised of six 50 kDa heterodimeric subunits (Gifford and Bewley, 1982, 1983). Similar to castor bean and angiosperms in general, conifer crystalloid proteins also consist of heterodimers linked by disulphide bridges (Gifford, 1988; Groome et al., 1991; Misra and Green, 1991; Green et al., 1991; Allona et al., 1992).

Other seed storage proteins including the 7S vicilin-like proteins (Flinn et al., 1991a; Allona et al., 1992; Newton et al. 1992) and low-molecular-weight globulins

(Gifford, 1988; Misra and Green, 1990; Flinn et al., 1991b; Allona et al., 1992; Hackman, 1993) have also been identified in seeds of several conifer species. This later group is believed to be related to the angiosperm albumins since both protein groups possess a dimeric structure consisting of a large and small subunit linked via a disulfide bridge. Interestingly, the low molecular weight globulins are also characterized by their relatively high arginine content (Allona et al., 1993). This high arginine content has been estimated as a significant nitrogen source in the form of free amino acids transported from megagametophyte to the embryo following germination. (King and Gifford, unpublished).

In gene expression studies, storage protein synthesis has been examined in developing seeds of white spruce (Leal and Misra, 1993), interior spruce (Flinn et al., 1991a, 1991b, 1993) and Norway spruce (Hackman et al., 1990; Hackman, 1993) (see Misra, 1995 for a review). In all cases examined, storage protein synthesis or mRNA accumulation began a few days following fertilization, increased dramatically during mid-embryogenesis and into the beginning of late embryogenesis, and then declined during the late stages of seed maturation (Leal and Misra, 1993a, 1993b). In conifers, the maximum expression of seed storage protein genes occurs during the embryonal mass stage, before the formation of the meristematic regions of the embryo. These results are in contrast to those described for angiosperms, where maximal storage protein expression occurs during cell expansion, a phase of embryogenesis after tissue differentiation has occurred. Based on these differences, Misra (1995) concluded that there must be a basic cellular difference in the pathways that regulate the synthesis of seed storage proteins during embryo development in angiosperms and gymnosperms.

Despite a current acceleration in the understanding of lipid storage synthesis in angiosperms (Murphy, 1993; Bewley and Black, 1995), any knowledge of these processes are virtually nonexistent in conifers. However, it has been established that seed conifer oil reserves are in the form of triacylglycerols and are typically high in linoleic acid (Firenzuoli et al., 1968; Durzan et al., 1971; Ching, 1973; Kovac and Vardjan 1981; Flinn et al., 1989; Janick et al., 1991). Furthermore, these triacylglycerol reserves are found in small (0.5 -2 μm) spherical oil bodies (Huang, 1992; Tzen et al., 1993). Recently, Lee et al. (1994) demonstrated that oil bodies of *Pinus ponderosa* contain oleosin, a structural protein on

the intracellular surface of the organelle. On the basis of a comparison of the deduced amino acid sequence of an oleosin cDNA from *Pinus ponderosa* and a number of angiosperms, Lee et al. (1994) concluded that gymnosperm and angiosperm oleosins constitute distinct evolutionary lineages. In terms of the ontogeny, the formation of conifer oil bodies is believed to follow the same mechanism proposed for angiosperms, where oil bodies are initially formed from the endoplasmic reticulum as triacylglycerol droplets, and then become engulfed by the endoplasmic reticulum and acquire surface coating oleosins (Murphy, 1993).

1.3. Lipid Catabolism

1.3.1. The Biochemistry of Lipid Catabolism in Conifers

Following seed germination, stored triacylglycerols in oil bodies are hydrolyzed to free fatty acids and glycerol via the action of lipase (Figure 1.3). Subsequently, the free fatty acids are transported to the glyoxysome where the enzymes of the β -oxidation and glyoxylate cycle pathways carry out the net conversion to succinate. Here enzymes such as CAT, ICL and MS carry out their metabolic roles. CAT is an enzyme of the β -oxidation pathway. It catalyzes the dismutation of toxic H_2O_2 produced as a result of the activity of acyl-CoA oxidase. ICL and MS participate in sequential reactions of the glyoxylate cycle, converting isocitrate into succinate and malate. Eventually the succinate will be converted to sucrose and this carbohydrate will be utilized as the primary energy source until photoautotrophic independence is obtained (Breidenbach et al., 1967; Cooper and Beevers, 1969; Hutton and Stumpf, 1969). In angiosperms this process of lipid mobilization has been well established (see Beevers, 1980; Trelease and Doman, 1984 for reviews). In conifers, significant progress over the last decade has suggested that a similar set of events are likely occurring during lipid hydrolysis and the conversion into sugar.

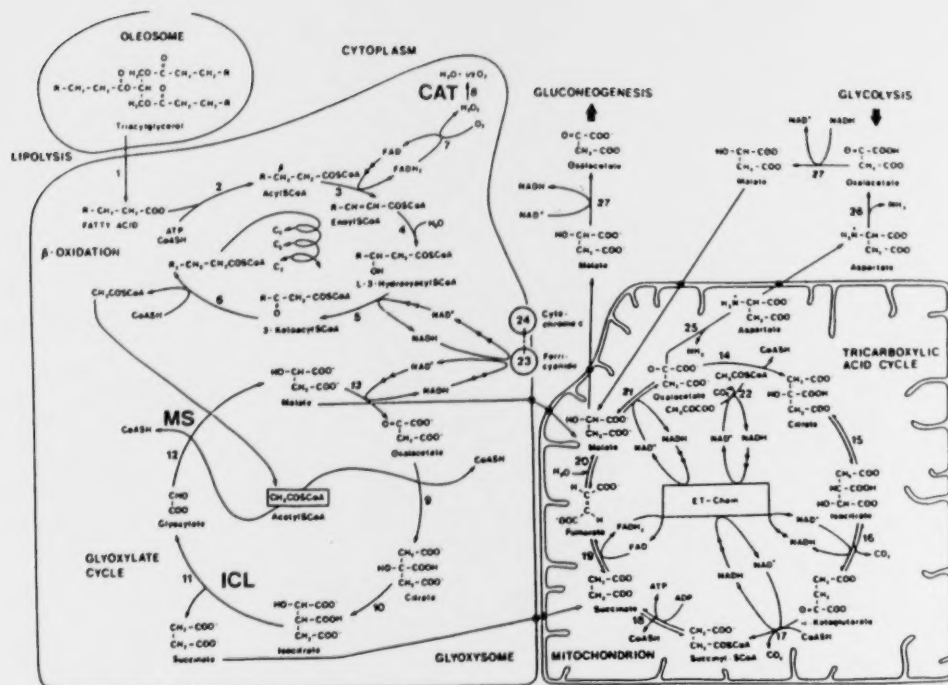


FIGURE 1.3. Biochemical pathway of lipid reserve mobilization in oil seeds. Enzymes numbered are: 1, lipase; 2, thiokinase; 3, acyl-CoA oxidase; 4, enoylhydratase; 5, hydroxyacyl dehydrogenase; 6, ketoacyl thiolase; 7, flavin oxidase; 8, CAT; 9, citrate synthase; 10, aconitase; 11, ICL; 12, MS; 13, malate dehydrogenase; 14, citrate synthase; 15, aconitase; 16, isocitrate dehydrogenase; 17, α -ketoglutarate dehydrogenase; 18, succinate thiokinase; 19, succinate dehydrogenase; 20, fumarase; 21, malate dehydrogenase; 22, pyruvate dehydrogenase; 23, ferricyanide reductase; 24, cytochrome b_5 ; 25, aspartate aminotransferase m; 26, aspartate aminotransferase c; 27, malate dehydrogenase. Adapted from Gietl (1992).

Lipid reserves in conifers seeds are stored as triacylglycerols in oil bodies in the megagametophyte and to a lesser extent in the embryo (Huang, 1992). Following germination, triacylglycerol reserves in both tissues are hydrolyzed by a lipase associated with the lipid bodies (Ching, 1968; Hammer and Murphy, 1993, 1994). Recently Hammer and Murphy (1994) identified a 64 kDa lipase protein in *Pinus edulis* seeds that was imported into the lipid body during germination. Cell-free enzyme activity assays and protein immunoblots also revealed that the lipase was developmentally regulated, reaching maximum levels concomitant with the rates of triacylglycerol utilization (Hammer and Murphy, 1994). Although the involvement of lipase enzyme activity was not examined, lipid hydrolysis during and following seed germination has been studied in a number of conifers (Ching, 1966; Durzan, 1971; Kao, 1973; Kovac and Wrischer, 1984; Groome et al., 1991; Janick et al., 1991).

Concomitant with the rates of lipid hydrolysis is an increase in the levels of activity of glyoxylate cycle enzymes in conifer seeds (Firenzuoli et al., 1968a, 1968b; Ching, 1963, 1966; Bilderback, 1973; Murray and Adams, 1980; Kovac and Wrischer, 1984; Noland and Murphy, 1984; Pitel and Cheliak, 1986). In relation to this, and as discussed in more detail below, the presence of glyoxysomes in conifers has been well established (Ching, 1970; Lopez-Perez et al., 1974). Overall, the existence of glyoxysomes and the presence of key glyoxylate cycle enzymes such as ICL and MS indicates that a functional glyoxylate cycle is operating in conifer seeds.

1.3.2. Regulation of Glyoxylate Cycle Enzyme Gene Expression

For angiosperms, a consensus has emerged whereby the expression of glyoxysomal enzymes such as CAT, ICL, and MS in oilseeds is primarily transcriptional; peak mRNA accumulation occurs about 24 h before peak protein and/or enzyme activity (Ni and Trelease, 1991; Thomas, 1993). In cucumber, a comparison of enzyme activities and the relative levels of translatable RNA for ICL and MS was carried using immunoprecipitation

with monospecific antibodies (Weir et al., 1980). Weir et al., (1980) concluded that the dramatic rise in ICL and MS enzyme activity in cucumber cotyledons following germination was preceded by, and presumably dependent upon, a comparable increase in the amounts of their translatable mRNAs. In relation to this, later studies have demonstrated that this increase in availability of translatable ICL and MS mRNAs is due to the synthesis of new mRNAs and not the differential translation of a pre-existing transcript pool. Smith and Leaver (1986) and Reynolds and Smith (1995) have both demonstrated that MS or ICL transcripts increase dramatically during post-germinative growth in cucumber, and that this rise is closely correlated with an increase in the accumulation of protein and enzyme activity. Allen et al. (1988) and Turley and Trelease (1990) also reported that changes in glyoxysomal enzyme transcripts preceded the corresponding peak of immunologically detectable protein levels in sunflower and cotton, respectively. In cotton, the steady-state level of mRNAs encoding CAT, ICL and MS exhibited a nonconcerted pattern of increase, but all peaked at least 24 h before peak enzyme activities in germinated seeds (Turley and Trelease, 1990). Evidence further supporting a theme of transcriptional regulation for glyoxysomal enzyme gene expression comes from run-on transcriptional assays for *Brassica napus* ICL and MS. By comparing the levels of transcripts on RNA blots to assays of nuclear RNA synthesis in isolated nuclei, Comai et al. (1989) concluded that ICL and MS are partly controlled by both transcriptional and post-transcriptional processes.

Compared to angiosperms, we know little of the regulation of glyoxylate cycle enzymes in conifers. Studies to date have, however, established that a similar dramatic increase in enzyme activity of glyoxylate cycle enzymes occurs in conifer seeds following germination (Ching, 1972). Interestingly, some evidence has been presented that the embryo axis may control glyoxylate cycle enzyme levels in the megagametophyte. Bilderback (1973), Murray and Adams (1980), and Groome et al. (1991) all demonstrated that removal of the embryo axis resulted in decreased levels of ICL activity in the megagametophyte of ponderosa pine and loblolly pine, respectively. At present, the form of control elicited by the embryo (i.e. signal) remains to be determined.

1.3.3. Classes of Higher Plant Peroxisomes

Since the focus of this thesis involves the developmental regulation of enzymes exclusively localized within the loblolly pine glyoxysome, in this section I will review some of the current understanding of the organelle in higher plants. For additional details about plant peroxisomes, the reader is referred to other reviews (Beevers, 1979; Huang et al., 1983; Trelease, 1984; Kindl, 1992; Olsen and Harada, 1993, 1995).

Peroxisomes occur in virtually all eukaryotic cells where they play critical roles in a variety of metabolic processes ranging from lipid mobilization to nitrogen transport (Tolbert, 1981; van den Bosch et al., 1992; Reddy and Mannaerts, 1994). Regardless of whether the organelle is from animals, plants, or yeast, all peroxisomes share a number of common characteristics. First, they are 0.2-1.8 μm in diameter and are bound by a single delimiting membrane. The matrix is usually finely granular, but occasionally crystalline or fibrillar inclusions are present (Huang et al., 1983). Second, they characteristically contain one or more H_2O_2 -producing flavin oxidases that together with CAT decompose H_2O_2 to oxygen and water (Tolbert, 1981). And finally these peroxisomes are nonautonomous in that they lack DNA, ribosomes, and an internal membrane system (Olsen and Harada, 1995). As a result, the constituents, which can include over 50 distinct enzymes, are synthesized on free polyribosomes and imported into the peroxisome from the cytosol (van den Bosch et al., 1992; Reddy and Mannaerts, 1994).

In higher plants there are at least three defined classes of peroxisomes that participate in distinct metabolic functions, usually at certain developmental stages of the plant's life cycle (Olsen and Harada, 1995). In oilseeds, such as conifers, peroxisomes are specifically named glyoxysomes to emphasize their possession of the glyoxylate cycle enzymes (i.e. ICL and MS) (Huang et al., 1983; Trelease, 1984). As discussed above these enzymes, along with the enzymes of the β -oxidation pathway, carry out the conversion of fatty acids to succinate. As a result, the glyoxysome and its constituents are responsible for the seedling's ability to mobilize lipids as a carbon source until photoautotrophic growth is obtained. Recently, functional glyoxysomes have also been

reported in senescent organs, where they are believed to be involved in utilization of membrane lipids (De Bellis et al., 1990, 1991; Gut and Matile, 1988; Graham et al., 1992). In conifers, glyoxysomes are the only class of peroxisomes that have been identified to date (Ching, 1970; Lopez-Perez et al., 1974).

Leaf-type peroxisomes are present in green tissues that are photosynthetically active and contain enzymes essential for the light-dependent reactions of photorespiration, including glycolate oxidase and hydroxypyruvate reductase (Tolbert, 1981; Huang et al., 1983). In conifers, leaf-type peroxisomes have yet to be identified. However, Stone and Gifford (unpublished) have demonstrated glycolate oxidase activity in germinated green embryos suggesting the presence of these organelles and a photorespiratory pathway.

The root nodules of certain nitrogen-fixing legumes contain specialized peroxisomes that possess enzymes involved in nitrogen transport (Schubert, 1986; Olsen and Harada, 1995). Here, via purine catabolism, and enzymes such as urate oxidase, fixed ammonia is assimilated and eventually converted to allantoin and allantoic acid. Although this pathway involves a complex set of reactions occurring in several cellular components, the last steps (i.e. conversion of urate to allantoin via urate oxidase) occur in peroxisomes.

Finally, the last class of peroxisomes are the unspecialized peroxisomes (Newcomb, 1982; Huang et al., 1983). These peroxisomes are typically smaller than glyoxysomes and leaf-type peroxisomes, and are present in plant tissues that are not photosynthetically active or do not contain storage lipids. As a result these peroxisomes have an undefined metabolic role. It has been speculated that these organelles may provide a general protection against overall oxygen toxicity in the cell (Olsen and Harada, 1995).

Over the past two decades, at least five models for peroxisome biogenesis have been proposed (see Trelease, 1984; Olsen and Harada, 1995 for reviews). Of the earlier models, based primarily on ultrastructural data, the endoplasmic reticulum model was the most generally accepted (Beevers, 1979; Huang et al., 1983; Trelease, 1984). Here peroxisome proliferation was the result of budding from the endoplasmic reticulum membrane. As a result, every peroxisomal matrix protein was synthesized on the rough endoplasmic reticulum, some were glycosylated, and all were then localized within the

newly-formed budded peroxisome. Later studies eventually demonstrated that this model of peroxisome biogenesis was invalid. Briefly, no clear luminal continuities were ever observed between the endoplasmic reticulum and peroxisomes (Osumi et al., 1975; Lazarow and Fujiki, 1985). Similarly, an electrophoretic analysis of the endoplasmic reticulum and peroxisome membrane proteins revealed no similarities (Kruse and Kindl, 1982). Today, the general consensus for peroxisome biogenesis is that the organelle results from the "growth and division" of pre-existing peroxisomes (Lazarow and Fujiki, 1985; Olsen and Harada, 1995). Here peroxisomal growth or enlargement is accomplished by expansion of the membranes and the post-translational import of peroxisomal membrane proteins. In cotyledonous oilseeds, such as cotton, membrane expansion is accomplished by the transfer of phospholipids from the depleting oil bodies to the glyoxysome (Chapman and Trelease, 1991). In conifers it remains to be determined if a similar mechanism of peroxisome biogenesis occurs in the germinated megagametophyte.

1.3.4. Peroxisomal Targeting Signals

Because peroxisomes do not contain their own genome, proteins destined for the organelle are nuclear encoded, synthesized on free polyribosomes in the cytosol, and post-translationally imported into the peroxisome (Lazarow and Fujiki, 1985; Subramani, 1992, 1993; Olsen and Harada, 1995). In contrast to precursor proteins destined for mitochondria, chloroplasts, or endoplasmic reticulum, the import of peroxisomal proteins is not generally associated with any detectable modification, such as proteolytic removal of a pre-sequence (Lazarow and Fujiki, 1985; Olsen and Harada, 1995). This implies that the signal for targeting proteins to the peroxisome resides in the mature polypeptide. To date, at least three peroxisomal protein targeting signals (PTS) have been localized to a number of different regions of peroxisomal proteins.

One, and generally considered the prototype PTS, is a carboxy-terminal tripeptide with a consensus sequence of serine-lysine-leucine (SKL) (Gould et al., 1987, 1988, 1989a). Originally this tripeptide motif was discovered as the peroxisomal targeting signal

of firefly luciferase (Gould et al., 1987). Gould et al. (1987) demonstrated that conservative substitution of each of the amino acids of the tripeptide still resulted in the correct targeting of luciferase or a nonperoxisomal passenger protein to the organelle. Since its first identification as a PTS, the SKL motif has been shown to be common to the C-terminus of several peroxisomal proteins from mammals, yeast, fungi, trypanosomes, and plants (Gould et al., 1988; Gould et al., 1989b; Keller et al., 1991). In plants, direct proof for the ability of this short peptide to be the PTS has been demonstrated several times. In transformed tobacco, Volokita (1991) demonstrated that the spinach C-terminal (ARL) was sufficient to target the passenger protein β -glucuronidase to the peroxisome. In subsequent studies both Olsen et al. (1993) and Trelease et al. (1994) demonstrated that a conserved variant of the SKL motif for *Brassica* or cotton ICL, respectively, was necessary and sufficient to target proteins to the peroxisome. At present it remains to be determined if a PTS functions in conifers.

Unlike most other peroxisomal proteins characterized by a C-terminal SKL PTS, thiolase from animals and plants possesses an N-terminal presequence (Gietl, 1990; Osumi et al., 1991; Swinkels et al., 1991; Preising-Müller, 1993; Olsen and Harada, 1995). Here a PTS with a consensus sequence of RLxxxxxQ/HL is located 5-11 amino acids from the amino-terminal protopeptide. Although the functionality of this PTS has not been examined in plants to date, in rat thiolase this PTS has shown to be necessary for import into peroxisomes (Swinkels et al., 1991).

Some peroxisomal proteins contain neither a carboxy-terminal SKL nor a cleavable amino-terminal signal. These peroxisomal proteins are targeting to the organelle by a third group of PTSs known as internal amino acid signals (Roggenkamp, 1992; Subramani, 1992; Olsen and Harada, 1995). To date the best characterized peroxisomal protein with this class of PTS is acyl-CoA oxidase from *Candida tropicalis* (Small et al., 1988; Kamiryo et al., 1989). Here at least two independent domains, including a large amino-terminal domain and an internal domain, are both sufficient to direct a reporter protein to the peroxisome. In plants, only one putative internal PTS has been identified. Behari and Baker (1993) demonstrated that the castor bean ICL C-terminal SKL motif was not required for peroxisomal import. Instead, a truncated version of the castor bean ICL

protein minus the last 37 amino acids of the C-terminal could still be imported into glyoxysomes *in vitro*. At present this conflicting interpretation of the role of the C-terminal SKL versus a potential internal PTS in the castor bean ICL protein remains to be determined.

In plants, a survey of carboxy-terminal sequences of all peroxisomal proteins has revealed that the SKL motif or a variant thereof is the most common PTS (Olsen and Harada, 1991). Over the past five years significant progress has been made in understanding the mechanisms which underlie peroxisomal targeting in higher plants (see Olsen and Harada, 1995 for review). In conifers, it now remains to be determined if peroxisomal destined proteins are targeted to the organelle by similar pathways.

1.4. The Present Study

Low seed germination rates and poor seedling vigor are major problems in several economically important coniferous species. In an attempt to eventually alleviate the recalcitrant nature of conifer seed germination this research was carried out to examine some of the basic biochemical and molecular events involved in seed germination and early seedling growth in loblolly pine. Previously, Groome et al. (1991) demonstrated that loblolly pine seed germination and post-germinative growth are accompanied by changes in the patterns of *de novo* synthesized protein subsets. To increase our understanding of the regulatory mechanisms involved in the expression of genes during these developmental stages, several key marker enzymes were examined. CAT, ICL and MS are glyoxysomal enzymes involved in the hydrolysis of lipid reserves in loblolly pine seeds following germination. Crucial to any studies of developmental regulation of these three enzymes is the availability of monospecific antibodies and cDNA clones. Research in this dissertation was designed to provide these molecular tools and to begin to examine the regulation of CAT, ICL and MS gene expression in loblolly pine seeds. CAT, ICL, and MS were all purified and polyclonal antibodies raised in rabbits. To isolate cDNA clones encoding these enzymes a megagametophyte cDNA library was constructed. Using either immunoscreening or the PCR, cDNA clones for ICL and CAT were isolated and

sequenced to entirety. Finally, the developmental regulation of all three enzymes was examined. In total, results presented in this thesis provide an outline of the general patterns of gene expression during loblolly pine seed germination and post-germinative growth, as well as a comparison of the developmental regulation of loblolly pine CAT, ICL, MS with their angiosperm counterparts.

2. MATERIALS AND METHODS

2.1. Chemicals and Equipment

Reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Fisher Scientific (Edmonton, AB, Canada), Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada) and BDH (Toronto, ON, Canada). Aqueous counting scintillant (ACS-II) was purchased from Amersham (Oakville, ON, Canada). Sequencing primers were purchased from Pharmacia (Montreal, QB, Canada) or were prepared by the DNA Synthesis Laboratory, Department of Biological Sciences, University of Alberta. Restriction endonucleases were purchased from Pharmacia, Gibco BRL (Gaithersburg, MD, USA), or Promega-Biotech (Madison, WI, USA). Agarose NA or low melting agarose was supplied by Pharmacia LKB Biotechnology (Uppsala, Sweden) and Sigma Chemical Co., respectively. Rabbit polyclonal antiserum was prepared by Animal Services (University of Alberta). Lithium chloride was supplied by Merck Chemical Co. (Toronto, ON, Canada). Media reagents were purchased from Difco Laboratories (Detroit, MI, USA). All radioisotopes including [^{35}S]-methionine, α [^{35}S]-dATP, α [^{32}P]-dCTP, and [^{14}C]-methylated molecular mass protein standards were obtained from Amersham. Antiserum to cotton CAT, ICL, and MS, as well as cDNA clones to all three cotton enzymes were generously provided by Dr. R. N. Trelease (Department of Botany, Arizona State University, Tempe, AZ, USA). SKL antiserum and *Yarrowia lipolytica* peroxisomal fractions were supplied by Dr. R. Rachubinski (Department of Anatomy and Cell Biology, University of Alberta, Edmonton, AB, Canada).

The LS6000TA Scintillation counter, Microfuge E®, J2-21M/E, Du-60 Spectrophotometer, and L8-60M Ultracentrifuge and rotors were from Beckman Instruments Inc. (Mississauga, ON, Canada). Eppendorf centrifuge 5415 was supplied by Brinkman Instruments Inc. (Westbury, NY, USA). Distilled and deionized water (conductivity ≥ 18 megohms/cm) was obtained from a Milli-Q Filtration Water System from the Millipore Corporation (Bedford, MA, USA). DNA gel photography was carried

out with a Polaroid DS-34 Direct Screen Instant Camera (Rexdale, ON, Canada) and a Mighty Bright Mighty Small Gel Illuminator purchased from Hoefer Scientific Instruments (San Francisco, CA, USA). The MLB-06 Miniature Horizontal Gel System was purchased from Tyler Research Instruments (Edmonton, Alberta, Canada). Columns for protein chromatography were purchased from Bio-Rad Labs Ltd. and a RediFrac Fraction Collector was supplied by Pharmacia LKB Biotechnology. Scanning densitometry was carried out on a Bio-Rad GS-670 Imaging Densitometer (Molecular Analyst PC Version 1.2 Software).

2.2. Seed Material and Germination

Loblolly pine (*Pinus taeda* L.) seeds (kindly provided by Westvaco, Summerville, SC, USA) were collected in 1984 from an open-pollinated, first-generation select tree (clone 11-9). Seeds were surface sterilized as previously described (Groome et al., 1991), except that H_2O_2 was omitted. Seeds were imbibed on sterile moist Kimpak (Seedboro Equipment, Chicago, IL, USA) at $2 \pm 0.5^\circ C$ in the dark for at least 30 d to overcome dormancy. After chilling, the non-dormant stratified seeds were surface-sterilized in 1% (v/v) NaOCl for 5 min and then transferred to autoclaved kimpak-lined germination trays. The trays were then placed in a germinator (Controlled Environments, Winnipeg, MB, Canada), continuously illuminated at $19 \mu mol m^{-2} s^{-1}$ at $30 \pm 0.5^\circ C$ for up to 14 d. Isolated megagametophytes and embryos were harvested and used fresh or frozen in liquid N_2 and stored at $-70^\circ C$. Lodgepole pine (*Pinus contorta* Dougl), white spruce (*Picea glauca* [Moench] Voss), and Douglas-fir (*Pseudotsuga menziesii* [Mirb] Franco) seeds were all obtained from the Pine Ridge Forest Nursery (Smokey Lake, AB, Canada) and were stratified and germinated under identical conditions as those described for loblolly pine.

Inbred castor bean seeds (*Ricinus communis* L. cv. Hale) were obtained commercially from Bothwell Enterprises (Plainview, TX, USA). After being soaked in deionized water for 1 h, seeds were decoated and imbibed on sterile, moist kimpak-lined germination trays at $25 \pm 0.5^\circ C$ in the dark for up to 6 d. Cotton seeds (*Gossypium hirsutum* L. cv. Deltapine 62) were kindly provided by Dr. R. N. Trelease (Department of

Botany, Arizona State University, Tempe, AZ, USA). Cotton seeds were soaked in deionized water with aeration for 4 h, then imbibed on sterile, moist kimpak-lined germination trays at $30 \pm 0.5^\circ\text{C}$ for time periods as indicated.

2.3. Enzyme and Protein Assays

All enzyme assays were conducted at room temperature (22°C) on a Beckman DU-65 spectrophotometer. Data presented for enzyme activity are the mean of three experiments, with enzyme assays carried out in triplicate for each of the replicates.

2.3.1. CAT Assay

CAT ($\text{H}_2\text{O}_2\text{:H}_2\text{O}_2$ oxidoreductase, EC 1.11.1.6) enzyme activity in cell-free extracts was assayed by the method of Lück (1963). The reaction mixture contained 135 mM potassium phosphate (pH 7.0), 0.096% (v/v) H_2O_2 , 0.0125% (v/v) Triton X-100, and enzyme in a final volume of 3 mL. Control reactions contained no H_2O_2 . The first order decomposition of H_2O_2 was monitored spectrophotometrically at an absorbance of 240 nm. One unit of CAT activity (kunit) was defined as the amount of enzyme that catalyzed the conversion of 1 mmol of H_2O_2 per min at 22°C (extinction coefficient: $40 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$; Aebi, 1974).

2.3.2. ICL Assay

ICL (threo-D-isocitrate glyoxylate-lyase, EC 4.1.3.1) activity was measured by the continuous assay according to Dixon and Kornberg (1959) and modified as described by Lamb et al. (1978). The reaction mixture contained 50 mM potassium phosphate (pH 7.2), 1.4 mM MgCl_2 , 1.62 mM phenylhydrazine, 1.1 mM DTT, 1 mM isocitrate, and enzyme to a final volume of 2.5 mL. The formation of glyoxylate phenylhydrazone was monitored as the change in absorbance at 324 nm. Controls contained no isocitric acid.

An extinction coefficient of $17 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Lamb et al., 1978) was used. One unit of ICL activity was defined as the amount of enzyme required to degrade 1 μmol of DL-isocitrate to glyoxylate and succinate per min at 22°C.

2.3.3. MS Assay

MS (L-malate glyoxylate-lyase [CoA-acetylating], EC 4.1.3.2) activity was assayed by titrating the resulting free sulfhydryl groups of coenzyme-A with Ellman's reagent (DTNB) as described by Cooper and Beevers (1969) with slight modifications. The complete reaction mixture contained 77 mM Tris-HCl (pH 8.0), 7.7 mM MgCl_2 , 1.5 mM DTNB, 0.18 mM acetyl-CoA, 20 mM glyoxylate (sodium salt), and enzyme in a final volume of 1.3 mL. The reaction was initiated by the addition of glyoxylate and the formation of the DTNB-CoA complexes was followed as increase in absorbance at 412 nm. Control reactions contained no glyoxylate. An extinction coefficient of $13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Silverstein, 1975). One unit of MS activity was defined as the amount of enzyme that released 1 μmol of CoA per min at 22°C.

2.3.4 Protein Assay

Protein was estimated by the method of Lowry et al. (1951) with BSA (Grade IV, Sigma Chemical Co.) as a standard. The total protein concentration of column chromatography effluents was monitored spectrophotometrically at 280 nm.

2.4. Protein Purification

2.4.1. Purification of CAT

Except where indicated, all procedures were carried out at 4°C. All buffers contained 10 mM DTT and 1 μM leupeptin (protease inhibitor; Sigma Chemical Co.) and were de-aerated overnight before use. The following buffers were used: buffer A, 10 mM

potassium phosphate, pH 7.0; buffer B, 50 mM Tris-HCl, pH 7.0, 100 mM NaCl; buffer C, 200 mM potassium phosphate, pH 7.0; buffer D, 10 mM Tris-HCl, pH 8.0, 5% (v/v) ethylene glycol.

Twenty g FW of 7 DAI isolated loblolly pine megagametophytes were ground to a powder in a cold mortar in liquid N_2 and re-extracted with 1:5 (w/v) cold acetone ($-20^{\circ}C$). For loblolly pine seeds, an initial extraction with acetone was found necessary to remove lipids that interfered with subsequent $(NH_4)_2SO_4$ protein fractionation's. The homogenate was centrifuged at 25,000 g for 30 min and the resulting precipitate was resuspended with a glass rod in buffer A. After homogenization in a Brinkman Polytron blender at high speed, the slurry was filtered through Miracloth (CalBiochem.) which had been presoaked in buffer A, and then centrifuged at 25,000 g for 30 min. The supernatant was then fractionated at $4^{\circ}C$ by the slow addition of solid $(NH_4)_2SO_4$. Proteins precipitating in the 30-70% saturation fraction were resuspended in buffer A and dialyzed overnight against three changes of the same buffer. Insoluble materials were removed by centrifugation at 14,000 g for 10 min, and the dialyzed sample was applied to a DE-52 cellulose column (1.5 x 25 cm; Whatman Biosystems Ltd., Kent, England) previously equilibrated with buffer A. After washing with five column volumes of the same buffer, a 100 mL linear gradient of NaCl (0-250 mM in buffer A) was applied at a flow rate of 30 mL h^{-1} . Peak CAT-activity fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation at $4^{\circ}C$ (80% saturation). The precipitate was resuspended in buffer B and applied directly to a Superdex-200 gel filtration column (3 mL min^{-1} ; Pharmacia LKB Biotechnology FPLC system) which had been equilibrated in buffer B. Peak CAT-activity fractions were pooled and precipitated with $(NH_4)_2SO_4$ (80% saturation) at $4^{\circ}C$ and the resulting pellet was resuspended in buffer A. The sample was dialyzed overnight against three changes of buffer A, centrifuged, and loaded directly onto a Bio-Gel HTP column (1 x 12 cm; Bio-Rad Labs Ltd.) equilibrated in buffer A. The column was washed with two column volumes of buffer A and then eluted with a 100 mL linear gradient of 10-200 mM potassium phosphate, pH 7.0, at a flow rate of 20 mL h^{-1} . Peak CAT-activity fractions were pooled and concentrated by $(NH_4)_2SO_4$ (100% saturation) at $4^{\circ}C$. The resulting pellet was resuspended in buffer C and loaded onto a phenyl-Sepharose CL-4B column (1

x 15 cm; Sigma Chemical Corp.) previously equilibrated with the same buffer. After washing with four column volumes of buffer C, CAT was eluted with a 100 mL reverse linear gradient (20 mL h^{-1}) consisting of 0.2-0 M potassium phosphate, pH 7.0, and 0-100% (v/v) ethylene glycol as described by Kunce et al. (1988). Peak CAT-activity fractions were pooled, concentrated and exchanged to buffer D using Centricon-30 Microconcentrators according to the manufacturer's instructions (Amicon Corp., Beverly, MA, USA), and stored at -70°C .

2.4.2. Purification of ICL

Except where indicated all procedures were carried out at 4°C . All buffers were de-aerated overnight and included 5 mM MgCl_2 , 1 mM EDTA, 1 μM leupeptin, 1 mM PMSF and 1 mM DTT.

Nine DAI isolated megagametophytes (20 g FW) were extracted in cold acetone (-20°C) and re-extracted in a blender in buffer A (50 mM Tris-HCl, pH 7.2) as previously described for CAT (section 2.4.1). Proteins precipitating in the 0-50% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction were resuspended in buffer A and dialyzed overnight against three changes of the same buffer. The sample was then loaded onto a DE-52 cellulose column (1.5 x 25 cm) previously equilibrated in buffer A. After the column was washed with four void volumes of buffer A, a linear gradient of NaCl (0-250 mM in buffer A) was applied at a flow rate of 30 mL h^{-1} . ICL activity eluted from this column as two peaks. The ICL A fractions eluting in the flow through (see section 3.2.1) were pooled, concentrated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation) and resuspended in buffer B (50 mM Tris-HCl pH 7.2, 150 mM NaCl). The sample was loaded directly onto a column of Sepharose CL-6B (2.5 x 90 cm; Sigma Chemical Co.) equilibrated and eluted in buffer B at a flow rate of 20 mL h^{-1} . ICL A-enriched fractions were pooled, concentrated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation) and dialyzed overnight in buffer C (10 mM potassium phosphate, pH 7.0). The sample was loaded onto a Bio-Gel HTP column (1 x 12 cm) and eluted at 20 mL h^{-1} with a linear gradient of 0-250 mM NaCl. Peak ICL A fractions were pooled, concentrated with $(\text{NH}_4)_2\text{SO}_4$ (80% saturation) and resuspended in buffer D (200 mM potassium phosphate,

pH 7.0). The sample was then loaded onto an octyl-Sepharose CL-4B column (1 x 15 cm; Sigma Chemical Co.) equilibrated in the same buffer and proteins eluted with a reverse linear gradient of 200-0 mM potassium phosphate, pH 7.0 and 0-100% ethylene glycol at 20 mL h⁻¹. Peak ICL A fractions were pooled, concentrated to 3 mL using Centricon-30 Micro-concentrators according to the manufacturer's instructions (Amicon Corp.), and stored at -70°C.

2.4.3. Purification of MS

Loblolly pine MS was purified by a modification of the procedure described by Trelease et al. (1987). All chromatographic steps were carried out at room temperature. Fifteen g FW of isolated 10 DAI megagametophytes were ground in liquid N₂ and extracted in cold acetone (-20°C) and re-extracted in buffer A (100 mM KPO₄, pH 7.2, 2 mM EDTA, 8 mM MgCl₂, 1 mM PMSF, 1 µM leupeptin, and 1 mM DTT) as previously described for CAT (see section 2.4.1). After centrifugation, the resulting supernatant was adjusted to 2 mM with sodium glyoxylate (buffer B) to prevent any possible enzyme aggregation (Köller and Kindl, 1977; Trelease et al. 1987), and proteins were then fractionated by the slow addition of solid (NH₄)₂SO₄. Protein precipitating in the 0-35% range of saturation were resuspended in buffer B and immediately applied to a Sepharose CL-6B column (2.5 x 90 cm; Sigma Chemical Co.) pre-equilibrated in buffer B containing 100 mM NaCl (20 mL h⁻¹). Peak MS activity fractions from gel filtration were pooled and applied to a column of DE-52 cellulose (1.5 x 25 cm) pre-equilibrated in buffer B at a flow rate of 30 mL h⁻¹. Flow-through MS fractions were then pooled and immediately loaded onto a phenyl-Sepharose CL-4B column (1 x 10 cm) previously equilibrated in buffer B. Again, MS did not bind to this matrix and flow through fractions were pooled and concentrated 30-fold by ultra-filtration with Centricon-30 Microconcentrators according to the manufacturer's instructions (Amicon Corp, Beverly, MA, USA.). The extract was finally applied to a Bio-Gel HTP column (1 x 10 cm; 20 mL h⁻¹ flow rate) pre-equilibrated in buffer B. Flow-through peak MS activity fractions were pooled, concentrated using ultra-filtration, brought to 5% (v/v) ethylene glycol and stored at -70°C.

2.5. Molecular Weight Determination

The apparent native molecular weights of purified enzymes were determined by gel filtration chromatography using a standardized Sepharose CL-6B column (2.5 x 90 cm; Sigma Chemical Co.). Standards used to calibrate the column included: thyroglobulin, 669 kDa; apoferritin, 443 kDa; β -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; and carbonic anhydrase, 29 kDa (Sigma Chemical Co.). The column was equilibrated and proteins eluted with 50 mM Tris-HCl, pH 7.5, 100 mM KCl at a flow rate of 20 mL h⁻¹. Three mL fractions were collected and assayed for enzyme activity. Blue dextran (Sigma Chemical Co.) was used to determine the void volume of the column.

2.6 Antibody Production

After collection of preimmune serum, female Flemish giant-French lop rabbits were immunized with approximately 30 μ g of purified protein in 0.75 mL PBS (PBS: 10 mM sodium phosphate, 1.8 mM potassium phosphate, 200 mM NaCl, 2.6 mM KCl, pH 7.4) and 0.75 mL Freund's complete adjuvant (Difco Labs). Antigen was injected subcutaneously and sub-scapularly at multiple sites on the back of the rabbit with a syringe coupler. Two separate booster injections every 28 d followed the same injection routes as the initial, except 20 μ g of antigen in 0.75 mL PBS and 0.75 mL Freund's incomplete adjuvant was used. Rabbits were exsanguinated 8-10 d after the third injection and blood treated as follows. Blood was incubated at 37°C for 2 h, then 4°C overnight to allow clotting. Red cells were removed by centrifugation at 12,000 g for 15 min in a clinical swinging bucket rotor (Sorvall GLC-1 General Laboratory Centrifuge, Markham, ON, Canada), and serum drawn off. The resulting antiserum was tested for titer by SDS-PAGE and protein blotting, aliquoted, and stored at -70°C in 0.01% (w/v) sodium azide.

2.7. Electrophoresis

2.7.1. SDS-PAGE

Sample preparation and SDS-PAGE were carried out as described by Laemmli (1970) using a Mini Gel System (Bio-Rad Labs Ltd.), with a 0.75 mm 7% or 12% (w/v) polyacrylamide (39:1 [w/v] acrylamide:bis-acrylamide) slab gel at 200 V. Following electrophoresis, proteins were visualized by either Coomassie blue R as described by Burk et al. (1983) or silver stained with a Bio-Rad silver stain kit according to the manufacturer's recommendations. The molecular masses of the proteins were determined by the method of Weber and Osborne (1969). Molecular mass markers included: phosphorylase b, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa (Bio-Rad Labs Ltd.). For fluorography, gels were treated with EN³HANCE (Dupont, Markham, ON, Canada), dried in a Bio-Rad Model 543 Gel Dryer, and exposed to X-OMAT AR film (Kodak, Rochester, NY, USA) at -70°C. [¹⁴C]-methylated molecular weight markers for labelled gels were: myosin, 200 kDa; phosphorylase b, 92.5 kDa; BSA, 69.0 kDa; ovalbumin, 46.0 kDa; carbonic anhydrase, 30.0 kDa; and lysozyme, 14.3 kDa (Amersham).

2.7.2. IEF/SDS-PAGE

Two-dimensional electrophoresis (IEF/SDS-PAGE) was carried out on a Mini Gel System (Bio-Rad Labs Ltd.) Samples, containing 9.5 M urea, were loaded at the cathode, in a tube gel containing 9.2 M urea, 4% (v/v) polyacrylamide (28.4:1.6 [w/v] acrylamide:bis-acrylamide), 1.6% (v/v) Pharmalyte (pH 5-8) and 0.4% (v/v) Pharmalyte (pH 3-10) (Sigma Chemical Co.). The cathodic (upper) and anodic (lower) solutions were 20 mM NaOH and 10 mM H₃PO₄, respectively, and both were de-aerated thoroughly before use. For the first dimension IEF electrophoresis, gels were pre-electrophoresed at 200 V (10 min), 300 V (15 min), and 400 V (15 min), samples loaded, and IEF performed at 500 V for 10 min followed by 750 V for 3.5 hr. After focusing, SDS-PAGE was

performed, under reducing conditions (+ 5% [v/v] β -mercaptoethanol), on a 1.5 mm 12% slab gel as described above (see section 2.7.1). Following electrophoresis, proteins were blotted to nitrocellulose (see section 2.9), or gels containing radio-labelled proteins were subjected to fluorography, dried, and exposed to X-OMAT AR (Kodak) film at -70°C .

2.7.3. Non-Denaturing IEF

Non-denaturing IEF was carried out on a Multiphor II Horizontal Electrophoresis System (Pharmacia LKB Biotechnology). Gels were cast using a Mini Casting Tray (Bio-Rad Labs Ltd.) and consisted of 5% (w/v) polyacrylamide (24.25:0.75 [w/v] acrylamide:bis-acrylamide), 5% (v/v) glycerol, and 5% (v/v) Pharmalyte (pH 5-8). Both the cathodic (20 mM NaOH) and anodic (50 mM acetic acid) solutions were freshly prepared and thoroughly de-aerated before use. Samples for IEF were prepared according to the procedures described by Kuncz and Trelease (1986) with modifications. Twelve isolated megagametophytes (0.15 g FW), frozen in liquid N_2 , were ground to a powder in a cold mortar. Samples were then extracted by continuing grinding in 0.25 mL of 100 mM Tris-HCl, pH 7.0, 20% (v/v) glycerol, 0.1 mM leupeptin, and 20 mM DTT. After microfugation at 14,000 g for 20 min, the supernatants (cell-free extracts) were diluted in extraction buffer so that they contained equal amounts of CAT activity. Samples were further diluted (1:1, v/v) with a solution of 80% (v/v) glycerol and 4% (v/v) Pharmalytes (pH 5-8). Electrophoresis was performed at 4°C and 500 V for 15 min followed by 750 V for 2 h. To determine the pI's of CAT isoforms, gels were rinsed briefly with deionized water and the pH gradient measured on the gel surface with a Multiphor Surface Electrode (Pharmacia LKB Biotechnology). For experiments with castor bean and cotton, horizontal non-denaturing IEF was carried out essentially as described for loblolly pine, with only a few modifications. One castor bean endosperm or one cotton cotyledon was extracted, and for castor bean, gels and buffers consisted of a 1:1 mix of 2.5% (v/v) Pharmalytes (pH 5-8) and (pH 6.5-9).

For protein blotting experiments, non-denaturing IEF electrophoresis was carried out on a vertical Mighty Small II Model SE 250 gel apparatus (Hoefer Scientific

Instruments) according to the procedures described by Robertson et al. (1987). Slab gels (1.5 mm) were cast in a Hoefer SE 215 Multiple Casting Chamber (Hoefer Scientific Instruments) and consisted of 5% (w/v) polyacrylamide (24.25:0.75 [w/v] acrylamide:bis-acrylamide), 10% (v/v) glycerol, and 5% (v/v) Pharmalyte (pH 5-8). Sample preparation and electrophoresis buffers were identical to those described for horizontal non-denaturing IEF (see above). Electrophoresis was performed at 4°C at 200 V for 2.5 h and 500 V for an additional 2.5 h. After electrophoresis, proteins were transferred electrophoretically to nitrocellulose as described below (see section 2.9).

2.7.4. Denaturing Urea IEF

Denaturing urea IEF was carried out on a Mighty Small II Model SE 250 gel apparatus (Hoefer Scientific Instruments) according to Robertson et al. (1987). Slab gels (1.5 mm) consisted of 8 M urea, 5% (w/v) polyacrylamide (24.25:0.75 [w/v] acrylamide:bis-acrylamide), 10% (v/v) glycerol, and 5% (v/v) Pharmalyte (pH 5-8). The cathodic and anode solutions were 50 mM NaOH and 25 mM H₃PO₄, respectively. Sample preparation was identical to that described for non-denaturing IEF (see section 2.7.3) except samples were diluted in an equal volume of lysis buffer (8 M urea, 2% [v/v] Pharmalyte [pH 5-8], 2% [v/v] Triton X-100, and 1% [v/v] β-mercaptoethanol). Samples were acid-loaded (anode) and underlaid into wells filled with 1:4 diluted lysis buffer. Focusing was performed at 4°C, at 250 V for 2.5 hr and 500 V for an additional 2.5 hr. After electrophoresis, proteins were transferred electrophoretically to nitrocellulose as described below (see section 2.9).

2.8. *In situ* CAT Activity Staining

After non-denaturing IEF electrophoresis, gels were negatively stained for CAT activity as described by Clare et al. (1984), but with a number of modifications. Gels were soaked in 100 mM potassium phosphate (pH 7.5) and 0.1 mg mL⁻¹ horseradish peroxidase

(EC 1.11.1.7, Type II; Sigma Chemical Co.) with gentle shaking at room temperature. After 1 h, H_2O_2 was added to a final concentration of 0.02% (v/v) and soaking was continued for 10 min. The gels were then rinsed quickly in several changes of deionized water, and incubated in 100 mM potassium phosphate (pH 7.5) containing 0.5 mg mL^{-1} DAB (Sigma Chemical Co.) until CAT activity bands were apparent, i.e. achromatic bands on a dark brown background. This gel was used as a negative in contact printing. This format results in maximal resolution provided by dark bands on a light background.

2.9. Protein (Western) Blotting

Following SDS-PAGE, or IEF/SDS-PAGE, gels were immersed for 20 min in chilled (4°C) transfer buffer (25 mM Tris, 192 mM glycine, and 20% [v/v] methanol). The subsequent electrophoretic transfer of proteins to nitrocellulose (Bio-Rad Labs Ltd.) was carried out overnight at 30 V (4°C) in a semidry Mini Trans-Blot Cell (Bio-Rad Labs Ltd.) according to the manufacturer's recommendations. The efficiency of protein blotting to the nitrocellulose membrane was determined by Ponceau S staining (Sambrook *et al.* 1989). Briefly, transferred polypeptides were visualized by staining membranes in a solution containing 2% (w/v) Ponceau S (Sigma Chemical Co.), 30% (w/v) trichloroacetic acid, and 30% (w/v) sulfosalicylic acid, for 10 min and destaining in several washes of deionized water. All subsequent immunodetection procedures were carried out at room temperature with gentle shaking as described by Kuncz and Trelease (1986). Membranes were blocked in Blotto (3% [w/v] Carnation nonfat dry milk, 150 mM NaCl, 20 mM Tris-HCl [pH 7.8]) for 1 h then incubated for 2 h with primary antiserum diluted in Blotto (see individual figure legends for dilutions). After washing for 1 h in several changes of Blotto, the membranes were incubated for 2 h with secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad Labs Ltd.) diluted (1:5000) in Blotto. The membranes were then washed for 1 h in several changes of Blotto and immunoreactive polypeptides visualized by staining with either NBT/BCIP or naphthol as-bi phosphate/fast red violet LB salt according to the supplier's instructions (Bio-Rad Labs Ltd.). Color development was halted by washing the membrane in stop solution (5% acetic acid)

followed by several washes with deionized water. Control reactions were performed using preimmune sera in place of antiserum, and an incubation step where the primary antiserum was omitted and only the secondary goat anti-rabbit IgG conjugated-alkaline phosphatase was used. No reactions were observed under these conditions.

After denaturing or non-denaturing IEF electrophoresis, gels were prepared for protein blotting as essentially described by Ni et al. (1990). Gels were briefly rinsed in water, then incubated for 1 h with gentle shaking in several changes of 100 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β -mercaptoethanol. Gels were then rinsed with transfer buffer (see above) and proteins electrophoretically transferred to nitrocellulose (Bio-Rad Labs Ltd.) at 80 V (4°C) overnight. Following blotting, incubation in antiserum and visualization of immunoreactive polypeptides were carried out as described above.

For some protein blotting experiments using SKL antiserum, immunodetection was carried out with a BM Chemiluminescence Western blotting Kit according to the manufacturer's instructions (Boehringer Mannheim [BM]). All steps were carried out at room temperature with gentle agitation. Following protein transfer, the nitrocellulose membrane (Bio-Rad Labs Ltd.) was washed twice (10 min each) in TBS (50 mM Tris-HCl, [pH 7.5], 150 mM NaCl), blocked for 1 hr in 1% (w/v) BM blocking solution, and then incubated with SKL antiserum (1:500 dilution) for 2 hr in 0.5% (w/v) BM blocking solution. Following primary antibody incubation, membranes were washed two times (10 min each) with TBST (TBS with 0.1% [v/v] Tween-20), two times (10 min each) with 0.5% (v/v) BM blocking solution, and then incubated for 2 hr with BM goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (1:2000 dilution) in 0.5% (w/v) BM blocking solution. After washing four times (15 min each) in TBST, immunodetection was carried out in the dark using BM Luminescence substrates according to the supplier's instructions (Boehringer Mannheim). Membranes were exposed to X-OMAT AR film (Kodak) at room temperature for up to 5 min.

2.10. Determination of Total Steady-State Protein

The total amounts of CAT, ICL and MS steady-state protein from loblolly pine seeds following imbibition was determined by slot blot analysis (Ni and Trelease 1991). Ten isolated megagametophytes from each stage following seed imbibition were homogenized in a cold mortar and pestle in 1 mL 50 mM sodium phosphate buffer (pH 7.5), containing 0.1 mM leupeptin. The homogenate was microfuged at 14,000 g for 20 min at 4°C and the resulting buffer-soluble supernatant fractions stored on ice or at -20°C. One microliter of this crude extract was diluted in 5 mL of 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 10 μ M leupeptin, and 200 μ l of this was applied to a slot and transferred to nitrocellulose (Bio-Rad Labs Ltd.) by passive diffusion in a Bio-Dot SF apparatus (Bio-Rad Labs Ltd.). Following blotting and immunodetection as described above, the relative levels of CAT, ICL and MS protein was determined and quantified by scanning densitometry, using the values obtained from a linear range (1 to 100 ng) of purified protein applied to each membrane.

2.11. Affinity Purification of Antibodies

The method of Smith and Fisher (1984) was used with the following modifications. After standard protein blotting and immunostaining (see section 2.8), blots were rinsed briefly in deionized water and the regions containing the protein bands of interest (as determined from the migration of the purified protein) were cut from the nitrocellulose membrane and placed together (up to 4 bands) into a 1.5 mL microfuge tube. IgGs were released from their antigen/membrane with four 1 min washes of 100 mM glycine-HCl (pH 2.3), 500 mM NaCl, 0.5% (v/v) Tween-20, 100 μ g mL⁻¹ BSA. Following each wash, eluates were pooled, then immediately neutralized by with 1 M Tris-HCL (pH 8.0) to a final concentration of 10 mM and then stored at 4°C. IgGs purified by this method were determined to be monospecific and were subsequently used for certain protein blotting experiments as specified in individual figure legends.

2.12. Immunotitration

Immunotitration experiments were carried out as described by Trelease et al. (1987), except samples were prepared in 50 mM sodium phosphate (pH 7.5), and 0.1 mM leupeptin. Megagametophyte cell-free extracts were incubated with various amounts of preimmune or antiserum for 1 h at room temperature, then overnight at 4°C. Samples were then microfuged (14,000 g, 30 min, 4°C) and the supernatants assayed for the relative amounts of CAT, ICL, and MS activities as compared to the samples incubated with preimmune serum or extraction buffer only. Control samples indicated no significant loss of enzyme activity with overnight incubation.

2.13. Isolation of Glyoxysomes

Glyoxysomes were isolated from germinated loblolly pine megagametophytes using a differential centrifugation procedure modified from that described by Maeshima and Beevers (1985). Megagametophytes (4 g FW) were chopped for 15 min with a razor blade in 8 mL grinding buffer (150 mM Tricine pH 7.5, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA [pH 7.5] and 16% [w/v] sucrose) in a Petri dish on ice. The crude homogenate was filtered through one layer of Miracloth (presoaked in grinding buffer) and centrifuged at 4°C at 270 g for 10 min in a Beckman JA-20 rotor. The supernatant was then centrifuged in the same rotor for 20 min at 10,000 g (4°C) to obtain a glyoxysome-enriched pellet (Chapman et al., 1989). The pellet was gently resuspended with a camel hair brush in 2 mL grinding buffer and layered onto a sucrose density gradient consisting of a 1.7 mL 60% (w/w) sucrose cushion, 6.8 mL 32-60% (w/w) linear sucrose gradient and a 1.7 mL top layer of 20% (w/w) sucrose. All sucrose solutions were buffered with 150 mM tricine (pH 7.5) and 1 mM EDTA (pH 7.5). After centrifugation at 50,000 g (20000 rpm) for 1.5 h at 4°C in a SW 40 rotor in a Model L8-M Ultracentrifuge (Beckman Instruments) at a setting of No. 7 acceleration and No. 7 deceleration, four protein bands were clearly separated within the gradient (Beevers, 1979). Fractions from each of the four protein bands were collected either from the top with a pulled Pasteur

pipette, or through the tube wall with a hypodermic syringe. The band position corresponding to the glyoxysome fraction was determined by assaying for ICL and CAT activity as marker enzymes. Glyoxysomes were broken osmotically by diluting peak ICL/CAT activity fractions with 2 volumes of 1 M KCl containing 1 mM EDTA (pH 7.5). For SDS-PAGE, broken glyoxysome fractions were concentrated by ultrafiltration in a Centricon-30 Microconcentrator according to the manufacturer's instructions, then proteins were dissociated by boiling for 10 min in Laemmli buffer (Laemmli, 1970).

Isolation of glyoxysomes from castor bean endosperm (approx. 13 g FW from 30 seeds) was accomplished with the same homogenizing buffer and sucrose gradients as described for loblolly pine megagametophytes.

2.14. Glycoprotein Staining Following SDS-PAGE

Visualization of glycoproteins with Schiff's reagent (Sigma Chemical Co.) on SDS-PAGE gels was carried out using the periodic acid-Schiff method as described by Fairbanks et al. (1971), with castor bean ricin as a control. After staining, gels were washed several times in 0.1% (v/v) sodium metabisulfite and 10 mM HCl until distinct pink bands on a clear background were obtained. Finally, gels were rinsed in 10% (v/v) acetic acid and dried.

2.15 Con A-Sepharose Binding Assay

For Con A-Sepharose (Sigma Chemical Corp.) experiments, 2 μ g of purified MS was applied to 1 mL of Con A-Sepharose matrix and mixed end-over-end on a nutator at room temperature. After 1 h, samples were microfuged at 14,000 g for 15 min and the resulting supernatant assayed for MS enzyme activity.

2.16. Glycosylation Assays

Optimal incubation times and enzyme concentrations for Endoglycosidase F (EC 3.2.1) and PNGase F assays with various substrates were determined empirically using RNase B as a control.

Endoglycosidase F (specific activity 4954 units mL⁻¹) assays were carried out as described by Elder and Alexander (1982) and modifications according to the manufacturer's instructions (Cedarlane Labs, Mississauga, ON, Canada). Briefly, the reaction mixture (10 µL), containing substrate in 100 mM sodium phosphate (pH 6.1), 50 mM EDTA, 1% (v/v) Nonidet P-40, 0.1% (v/v) SDS, 1% (v/v), and β-mercapthoethanol, was denatured by boiling at 95°C for 2 min prior to the addition of 5 units of Endoglycosidase F. The reaction mixtures were then incubated for 3 h at 37°C, terminated by diluting 1:1 (v/v) in Laemmli buffer (Laemmli, 1970), boiled for 5 min, and stored at -20°C until SDS-PAGE analysis. The deglycosylation of RNase B (Sigma Chemical Co.) to RNase A was used as a control reaction.

PNGase F assays (specific activity 1 x 10⁶ units mL⁻¹) were carried out according to the supplier's recommendations (New England Biolabs, Beverly, MA, USA), with RNase B as a control. Prior to incubation with PNGase F, substrates were denatured by boiling at 95°C for 10 min in 0.5% (v/v) SDS and 1% (v/v) β-mercapthoethanol. Reactions (20 µL) were carried out by the addition of 1000 units PNGase F in 50 mM sodium phosphate (pH 7.5) for 1 or 20 h at 37°C, terminated by diluting 1:1 in Laemmli buffer (Laemmli, 1970), boiled for 5 min, and stored at -20°C until SDS-PAGE.

2.17. *In vivo* Protein Labelling

Ten freshly harvested megagametophytes (at the various developmental stages examined) were labelled with 3.8 GBq [³⁵S]-methionine (40 PBq mol⁻¹) for 3 h at 30°C. Tissue was then rinsed with deionized water and buffer-soluble proteins extracted in a cold mortar in 1 mL 50 mM NaPO₄ (pH 7.5), containing 0.1 mM leupeptin, 2 µg mL⁻¹

apoprotinin, and 1 mM iodoacetamide (protease inhibitors, Sigma Chemical Co.) at 4°C. Radioactive protein incorporation was determined by TCA precipitation (Gifford and Bewley, 1984).

2.18. RNA Isolation

All glassware and aqueous solutions required for RNA preparation were first treated with 0.1% (v/v) DEPC (Sigma Chemical Corp.) at 37°C overnight and subsequently autoclaved for 20 min. Total RNA was isolated from castor bean endosperm or loblolly pine embryos and megagametophytes at various stages of development by either a modification of the procedure of Verwoerd et al. (1989) or using a Promega RNeasy total RNA isolation system according to the manufacturer's instructions (Promega-Biotech.).

For small scale preparation of total RNA (Verwoerd et al., 1989), 200 mg of frozen material was ground to a fine powder with a steel bar in a sterile eppendorf tube. After grinding, 0.5 mL of hot extraction buffer (phenol-100 mM LiCl, 100 mM Tris-HCl [pH 8], 10 mM EDTA, 1% [w/v] SDS [1:1], at 80°C) and 0.25 mL chloroform-isoamyl alcohol (24:1) was added and vortexed vigorously for at least 1 min. Following microfugation (14,000 g at 4°C for 5 min), the aqueous phase was removed and diluted 1:1 with 4 M LiCl. RNA was precipitated by overnight incubation at -20°C and collected the next day by centrifugation. RNA pellets were resuspended in 0.25 mL deionized water, and RNA reprecipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 95% (v/v) ethanol at -20°C overnight. After centrifugation, RNA pellets were washed two times in 70% (v/v) ethanol and resuspended in 20 µL deionized water.

Poly(A)⁺ RNA was separated and collected using a PolyATract mRNA isolation system III according to the manufacturer's instructions (Promega-Biotech.). All RNA samples were stored as an ethanol precipitate at -70°C, quantified spectrophotometrically at 260/280 nm, and their integrity determined by agarose gel electrophoresis and ethidium bromide staining.

2.19. Translation *in vitro*

Just before translation, mRNA samples were heated for 5 min at 65°C and then cooled in ice water as described by Rachubinski et al. (1984). Cell-free protein translation was performed at 30°C for 1 hr in a RNA-dependent, nuclease-treated rabbit reticulocyte lysate system (Promega-Biotech.) as described by the supplier. Each 50 μL assay contained 2 μL of 10 $\mu\text{g mL}^{-1}$ poly(A)⁺ RNA, 4 μL [³⁵S]-methionine (40 PBq mol⁻¹) and 43 μL of translation mixture (Promega-Biotech.). The incorporation of radioactivity into proteins was determined by TCA precipitation according to the manufacturer's recommendations (Promega-Biotech.). Translation products were aliquoted and stored at -70°C until subsequent SDS-PAGE and/or IEF/SDS-PAGE and fluorography, or immunoabsorption with CAT, ICL, or MS antiserum.

2.20. *In vivo* and *in vitro* Immunoprecipitation

Immunoprecipitation of *in vivo* and *in vitro* labelled proteins was carried out essentially as described by Harlow and Lane (1988) and Turley and Trelease (1987). All immunoprecipitation and washing procedures were optimized for enzyme yield and to give minimal sample contamination. All steps were performed at 4°C and centrifugation at 14,000 g unless otherwise stated.

Aliquots of *in vivo* labelled and *in vitro* translation protein reactions were diluted to 1 mL with buffer A (50 mM Tris-HCl, pH 7.5, 1% [v/v] Triton X-100, 150 mM NaCl, 20 mM methionine, 1 mM iodoacetamide, 3 mM EDTA, 2 $\mu\text{g mL}^{-1}$ aprotinin, 1 mM PMSF, 25 $\mu\text{g mL}^{-1}$ leupeptin), microfuged for 15 min, and the supernatants brought to 1M NaCl by the addition of 50 mg solid NaCl. The mixtures were then precleared by incubating them with preimmune serum drawn from the same antigen immunized rabbit. Briefly, the diluted lysate samples were incubated for 1 h on ice with 50 μL of preimmune serum, then incubated for another 30 min on ice with 50 μL of a 10% suspension of *S. aureus* Cowan I cells (Sigma Chemical Co.) pre-washed three times in buffer A. Each

mixture was microfuged for 15 min and the supernatant removed for subsequent antigen immunoprecipitation. The supernatants were incubated with antiserum for 1 h at 25°C, then overnight on a nutator at 4°C. In a modification designed to increase yield, proteins synthesized *in vitro* were immunoprecipitated as described above, except 2-5 µg of purified protein was included in the mixture to act as a carrier. Immune complexes were collected by the addition of 20 µL of 10% *S. aureus* Cowan I cells pre-washed three times in buffer A. After a 1 h incubation at room temperature with end-over-end mixing, cells were collected by centrifugation and washed five times in buffer B (same as buffer A except 0.1% [v/v] Triton X-100 was used) and three times in buffer C (same as buffer A except Triton X-100 was omitted). Finally, the immune complexes were washed and dissociated by boiling in 50 µL Laemmli buffer (Laemmli, 1970), cells removed by centrifugation, and the resulting immunoselected proteins analyzed by SDS-PAGE and fluorography.

2.21. RNA Gels and Blotting Analysis

Total RNA (10 µg) was denatured in 50% (v/v) formamide and 6% (v/v) formaldehyde at 65°C for 15 min and electrophoresed on a 1.2% (w/v) agarose gel containing 0.66 M formaldehyde in 1 x MOPS buffer (20 mM MOPS [pH 7.5], 5 mM sodium acetate, 1 mM EDTA) as described by Sambrook et al. (1989). After electrophoresis, formaldehyde was removed from the gel by two brief washes with deionized water (5 min each), followed by two 25 min washes with 10 x SSC (1 x SSC = 150 mM sodium chloride, 15 mM sodium citrate) at room temperature. The RNA was then passively transferred to Zeta probe nylon hybridization membrane (Bio-Rad Labs Ltd.) by capillary flow of 10 x SSC overnight (Sambrook et al., 1989). The filter was washed in 2 x SSPE (1 x SSPE = 150 mM sodium citrate, 10 mM NaH₂PO₄·H₂O, 1 mM EDTA·Na₂ [pH 7.4]), air dried (30 min) and then the RNA fixed by baking in a vacuum oven at 80°C for 30 min (Johns Scientific Inc. Cornelius, OR, USA). Prior to probing, membranes were presoaked in 2 x SSPE for 5 min then prehybridized at 42°C for ≈3 h in a solution (20 mL) containing 50% (v/v) formamide, 5 x SSPE, 5 x Denhardt's solution (50

x Denhardt's = 1% [w/v] Ficoll, 1% [w/v] PVP, and 1% [w/v] BSA), 1 % (v/v) SDS, 10% (w/v) PEG, and $10 \mu\text{g mL}^{-1}$ denatured salmon sperm DNA in a Tyler Hybridization Incubator Model HI-12000 (Tyler Research Instruments). Hybridization was then carried out at 42°C overnight in the same solution as prehybridization except with the addition of 10^6 dpm mL^{-1} $\alpha[^{32}\text{P}]$ -labeled cDNA probe (in 10 mL). After hybridization, the membrane was washed twice for 15 min at room temp in 2 x SSPE, then twice at 65°C in 2 x SSPE, 2% SDS for 45 min, and finally twice at room temperature in 0.1 x SSPE for 15 min each. For ICL RNA blotting experiments the final wash was carried out under high-stringency conditions (twice [15 min each] 0.1 x SSPE at 65°C) for gene-specific hybridization. Blots were wrapped in Saran Wrap and exposed to X-OMAT AR film (Kodak) at -70°C with intensifying screens for various periods of time (see individual figure legends). The relative intensity of transcript levels during various stages following seed imbibition was quantified by scanning densitometry.

For hybridization probes, gel-purified cDNAs inserts were radiolabeled to a specific activity $\geq 10^8 \text{ dpm } \mu\text{g}^{-1}$ DNA with $\alpha[^{32}\text{P}]$ -dCTP ($110 \text{ TBq mmol}^{-1}$, 370 MBq mL^{-1}) by random priming using a Pharmacia Oligolabeling kit according to the manufacturer's recommendations. Size determination of transcripts was determined using the relative migration of molecular size markers (RNA ladder; Bethesda Research Laboratories) run on the same gel. After electrophoresis, this lane was submerged in a 10 mg mL^{-1} ethidium bromide solution for 30 min then destained in several changes of deionized water.

2.22. Slot and Dot Blot RNA Analysis

For slot and dot blots, 3 μg of total RNA was either alkaline denatured with 10 mM NaOH, 1 mM EDTA (pH 7.5) or denatured by incubation at 60°C for 15 min in 50% (v/v) formamide, 6% (v/v) formaldehyde, and 0.5 x MOPS buffer. RNA was vacuum filtered in triplicate onto a Zeta-Probe Nylon membrane using a Bio-Dot SF apparatus according to the manufacturer's instructions (Bio-Rad Labs Ltd.). For all blotting experiments, controls were rRNA, tRNA, and no RNA loaded, and any ensuing signals were subtracted as a background level for all blots. Membranes were washed briefly in 2 x SSC and 0.1% (v/v) SDS, air dried and then vacuum baked at 80°C for 30 min. Procedures for probe preparation, prehybridization, hybridization, and washing (under high-stringency conditions) were identical to that described above for RNA northern blotting experiments. After overnight exposure at -70°C, the relative intensity of transcripts was determined by scanning densitometry.

2.23. Construction of Megagametophyte and Cotyledonary cDNA Libraries

For cDNA library construction, poly(A)⁺ RNA was purified from total RNA isolated from 8 DAI loblolly pine megagametophytes using a PolyAtract mRNA isolation system III. Prior to cDNA synthesis, the quality of this mRNA preparation was first determined by RNA gel electrophoresis and then *in vitro* translation in a nuclease-free, rabbit reticulocyte lysate (Promega-Biotech.) where the resulting protein products were analyzed by SDS-PAGE and fluorography. Construction of a 9 DAI cotyledon (embryo) specific cDNA library was carried out exactly as described below for the megagametophyte library.

First and second strand cDNA synthesis was carried out using 5 μg of Poly(A)⁺ RNA with a ZAP-cDNA Synthesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Following second strand synthesis, cDNA was

extracted once in phenol-chloroform (1:1 [v/v]; pH 7.5), and precipitated overnight in 3 M sodium acetate (pH 5.2) and 2 volumes 100% (v/v) ethanol at -20°C. After microfugation at 14,000 g for 60 min (4°C), the cDNA pellet was washed once in cold (-20°C) 80% (v/v) ethanol, dried at room temperature and resuspended in deionized nuclease-free water. For both first and second strand synthesis, a small aliquot of each reaction was removed and analyzed by TCA precipitation, and alkaline agarose gel electrophoresis to determine the size distribution of cDNA products and the presence of any possible secondary structures, i.e., hairpinning (Sambrook et al. 1989).

Following analysis of cDNA synthesis reactions, cDNA ends were filled with Klenow fragment, and EcoR 1 adaptors ligated to the blunt ends at 4°C overnight according to the supplier's instructions (Stratagene). EcoR I adaptors were then kinased and 3' terminal adaptors released by Xho I digestion, again according to the suppliers instructions. Following digestion, unligated adaptors were removed, and cDNA size-fractionated by centrifugation on a Sephacyl S-400 column (Promega-Biotech.) in a swinging bucket rotor (Sorvall GL-C1 Clinical Centrifuge) at 400 g (1400 rpm). The column was then washed twice in one void volume of 1 x STE (100 mM NaCl, 20 mM Tris-HCl [pH 7.5], and 10 mM EDTA) and all eluted cDNA fractions analyzed by 5% (v/v) non-denaturing PAGE in a Bio-Rad Mini Gel System (Davis et al. 1986). Following electrophoresis, the portion of the gel containing the DNA molecular mass markers (Gibco BRL) was cut and stained with 10 mg mL⁻¹ ethidium bromide. The remaining gel with cDNA fractions was wrapped in Saran Wrap and exposed overnight at -70°C to X-OMAT AR film (Kodak) with intensifying screens. Those fractions with high molecular weight cDNA species (≥ 500 bp) and no free adaptors were collected, extracted once with phenol-chloroform (1:1 [v/v]), microfuged, and the resulting aqueous layer re-extracted once again with an equal volume of chloroform. cDNAs were then precipitated overnight at -20°C by the addition of an equal volume of 100% (v/v) ethanol. Following microfugation at 14,000 g for 60 min (4°C), the relative amount of DNA in the resulting pellet was measured by radioactivity with a Geiger-counter. The cDNA pellet was resuspended in deionized water and quantified by an ethidium bromide plate assay according to the manufacturer's instructions (Stratagene).

Ligation of 100 ng of cDNA into UNI-ZAP XR vector arms was carried out as recommended by the supplier using a test insert DNA as a control (Stratagene). The ligation reaction was incubated at 4°C for 48 hr, and the entire library was then packaged *in vitro* using Gigapack II Gold packaging extract according to the manufacturer's instructions (Stratagene). The library was then plated and titered with the *E. coli* line XL1-Blue MRF' on NZY plates (125 mM NaCl, 16 mM MgSO₄, 0.5% [w/v] yeast extract, 1% [w/v] casein hydrolysate, and 1.5 % [w/v] agar) containing 3 mM IPTG and 5 mg mL⁻¹ X-Gal to determined the percentage of recombinant versus nonrecombinant clones in the library. For library amplification, 20 x 150 mm plates (50,000 pfu/plate) were overlayed with 8 mL of SM buffer (145 mM NaCl, 16 mM MgSO₄, 50 mM Tris HCl [pH 7.5], and 0.01% [w/v] gelatin) and incubated overnight with gentle rocking to allow the phage to diffuse. The bacteriophage suspension buffer was collected from each plate, pooled, and chloroform added to a final concentration of 5% (v/v). Cell debris was removed by centrifugation and the resulting library was checked for titer according to the manufacturer's instructions (Stratagene).

2.24. ICL Immunoscreening of cDNA Library with ICL Antiserum

A total of 2.5×10^5 plaques (50,000 pfu 150 mm plate⁻¹) from the megagametophyte λ ZAP cDNA library were transferred to nitrocellulose filters (Nitroplus MSI, Westboro, MA, USA) and screened with loblolly pine ICL antiserum using a *picoBlue* immunoscreening kit according to the manufacturer's instructions (Stratagene), with only a few modifications. Following infection and plating, plates were incubated at 42°C until plaques were visible (≈ 3.5 hr). The top agar was then overlayed with nitrocellulose filters that were pre-soaked in 10 mM IPTG for 30 min and air dried on Whatman 3MM paper. The plates were then incubated at 37°C for 3.5 hr, then overnight at 4°C. Filters were marked with India ink, and immunodetection was carried out according to the supplier's instructions using loblolly pine ICL antiserum and goat anti-rabbit conjugated alkaline phosphatase with NBT/BCIP as substrates (Stratagene). Positives plaques were selected and purified through an additional three rounds of

screening. cDNA inserts from positive plaques were rescued from the UNI-ZAP XR vector by *in vivo* excision with ExAssist helper phage and SOLR *E.coli*, to form packaged pBluescript (SK-) phagemid, all according to the manufacturer's instructions (Stratagene). The resulting excised pBluescript (SK-) phagemids were then selected on the basis of ampicillin resistance, DNA isolated (Sambrook et al., 1989), and cDNA inserts excised by EcoR I/Kpn I digestion and analyzed by horizontal gel electrophoresis. 5' and 3' ends of selected clones were analyzed by DNA sequencing, and the complete nucleotide sequences (coding and noncoding strands) of the ICL clones designated PtBs ICL 8 and PtBs ICL 12 were determined (see section 2.26).

2.25. Isolation of Partial CAT cDNA

Small scale phage DNA isolation from a liquid lysate of the λ ZAP megagametophyte library was carried out as essentially described by Sambrook et al. (1988) with only a few modifications. 10^8 plaques of the megagametophyte cDNA library were amplified by vigorous shaking (250 rpm) in 50 mL NZY until lysis occurred (≈ 8 hr). The phage were centrifuged at 27,000 rpm for 1.5 hr in a SW 28 rotor in a Model L8-60M Ultracentrifuge (Beckman Instruments). The resulting phage pellet was resuspended in 200 μ L of 50 mM Tris HCl (pH 7.5) and extracted once in Tris-buffered phenol (pH 8.0) and microfuged at 14,000 g for 2 min at room temperature. Following centrifugation the aqueous phase was removed and re-extracted two more times with phenol, then twice with chloroform. DNA was then precipitated overnight at -20°C with the addition of 0.1 vol. 3 M sodium acetate (pH 4.8) and 2 vol. 100% (v/v) ethanol. Following microfugation, the DNA pellet was resuspended in TE containing $10 \mu\text{g mL}^{-1}$ RNase A and analyzed and quantified by horizontal gel electrophoresis.

To isolate a cDNA encoding loblolly pine CAT, the above phage DNA preparation from the megagametophyte cDNA library was used as a template for PCR (Lee and Caskey, 1990) with the degenerate primers designated pCAT 1, pCAT 2, pCAT 3, and pCAT 4 (Table 2.1). All four primers were designed based on highly conserved domains of plant catalases corresponding to the amino acid regions in the 5' half of the gene:

MDPYKHRP (AA 1-8, pCAT 1 [sense]) and NYPEWKL (AA 263-269, pCAT 3 [antisense], and the 3' half of the gene: VKFHWKP (AA 222-228, pCAT 4 [sense]) and LNVRPSI (AA 486-492, pCAT 2 [antisense]) (Ni et al., 1990; Isin and Allen, 1991; Ni and Trelease, 1991; Chevalier et al., 1992; Drory and Woodson, 1992). PCR reaction mixtures contained 250 ng of template DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1% (v/v) Triton X-100, 0.2 mM of each dNTP (Pharmacia), 100 pmol of each oligonucleotide sense and antisense primer (i.e., pCAT 1 and pCAT 3, or pCAT 2 and pCAT 4), and 2.5 units of *Taq* DNA polymerase (Promega-Biotech) in a final volume of 50 μ L. PCR was carried out in a PTC-150-16 Minicycler (MJ Research) under the following conditions: the reaction mixture was held at 94°C for 5 min to denature, ramped to 50°C at 1°C 4 s⁻¹, held at 50°C for 1 min, ramped to 72°C at 1°C 4 s⁻¹, held at 72°C for 2 min, then brought to 94°C for 1 min. The cycle was repeated 25 times, ending with a 5 min extension period at 72°C. PCR products were analyzed by agarose gel electrophoresis, purified using GeneClean glass bead solution (Biolabs 101, Mississauga, ON, Canada), and ligated into the pGEM-T Vector according to the manufacturer's instructions (Promega-Biotech.). The pGEM-T vector has a 3' terminal thymidine nucleotide on both ends of the insertion site. This allows for the efficient ligation of PCR products to the additional adenosine nucleotides the 3'-terminal of double stranded products (Clark, 1988). Plasmid DNA containing the PCR generated cDNAs were analyzed by restriction endonuclease mapping and both strands sequenced as described below (see section 2.26). To discern any mutations in the sequences because of reading mistakes of the *Taq* polymerase during the PCR, sequences from the 5' CAT cDNA PCR product were confirmed twice with fragments obtained from two independent PCR amplifications.

2.26. DNA Sequencing

Double stranded sequencing was performed by the dideoxy-chain termination method using a T7 sequencing kit according to the manufacturer's instructions (Pharmacia) (Zhang et al., 1988), and specific custom-synthesized oligonucleotide primers

designed from the previous round of sequence data (Table 2.1). For template preparation, plasmid DNA was prepared by the alkaline lysis method (Sambrook et al., 1989). Sequencing was performed using a Sequi-Gen Nucleic Acid Sequencing Cell and α [^{35} S]-dATP (37 TBq mmol $^{-1}$) according to the supplier's recommendations (Bio-Rad Labs Ltd.). Following sequencing, the gel was transferred to Whatman 3MM paper, dried at 80°C for 1 hr on a Bio-Rad Model 583 Gel Dryer, and then exposed to X-ray film (Kodak X-OMAT AR) with intensifying screens at room temperature overnight. Analysis of DNA and deduced amino acid sequences was carried out using Sequaid DNA and PC/Gene (Clustal) Software and the National Center for Biotechnology Information BLAST E-MAIL server.

2.27. Other DNA Manipulations

All standard DNA manipulations such restriction endonuclease assays, colony and plaque growth, DNA preparation and electrophoresis, and overall general procedures such as storage were all carried out according to Sambrook et al. (1989). Small scale λ DNA preparations were carried out according to Grossberger (1987).

TABLE 2.1. Primers used for PCR and sequencing

Primer	Use	Sequence (5' to 3')
pCAT 1	PCR	ATGGATCCTTACAAGCA(TC)CGTCC
pCAT 2	PCR	TA(AC)ATGCTTGGTCTCACGTT(CG)AG
pCAT 3	PCR	AG(CT)TTCCA(CT)TCAGG(AG)TAGT
pCAT 4	PCR	GTGAAATTCCA(CT)TGGAA(AG)CCCAC
psCAT 5 21	sequencing	²⁰² GGAGCTAGTGCGAAGGG ²¹⁸
psCAT 5 22	sequencing	⁵⁶⁵ GAAAGGTGAACATATGC ⁵⁴⁹
psJCL 8 4	sequencing	¹¹³ GCGAGGTTTGAGGCAGAG ¹³⁰
psJCL 8 5	sequencing	²⁰⁰² CATAATTCCCCTCTCTAC ¹⁹⁸⁵
psJCL 8 9	sequencing	³⁹³ GGATGGCAATGTTCATCC ⁴¹⁰
psJCL 8 10	sequencing	¹⁵⁸¹ CGTATGCAAGCATCCCTC ¹⁵⁶⁴
psJCL 8 13	sequencing	⁷⁷⁴ GGTTGCTGCCCAGGCTCC ⁷⁹⁰
psJCL 8 14	sequencing	¹²⁸⁹ GGCCCCAAAAGCCCATCC ¹²⁷³
psJCL 8 17	sequencing	¹¹⁵⁵ GGCTAAGGAATTGGCTG ¹¹⁷¹
psJCL 8 18	sequencing	⁹⁶² CTGGCCATAGCTCGTGC ⁹⁴⁶
psJCL 8 23	sequencing	¹⁴⁷¹ GCACGGTTAGGTTATTGC ¹⁴⁸⁸
psJCL 8 24	sequencing	⁴⁸⁹ GTGCTCAACCTTGTTCCC ⁴⁷²
psJCL 12 6	sequencing	¹⁸⁴ GGCCATACACTGCAAAGG ²⁰¹
psJCL 12 7	sequencing	²⁰⁴⁷ GCTGCAGCAAAAGTTTGC ²⁰³⁰
psJCL 12 11	sequencing	³⁴² GCCATGATGGCCAAGTAC ³⁵⁹

<i>psJCL</i> 12 12	sequencing	¹⁸⁸⁷ CCAACAGGAATCCATCC ¹⁸⁷¹
<i>psJCL</i> 12 15	sequencing	⁶⁷¹ GCACATTGAGGACCAGG ⁶⁸⁷
<i>psJCL</i> 12 19	sequencing	⁹⁹⁶ GGTATGGCCAATTTGAG ¹⁰¹²
<i>psJCL</i> 12 20	sequencing	¹²⁴⁶ CCCCTGAGAATAGCAGC ¹²³⁰
<i>psJCL</i> 12 25	sequencing	¹⁵⁹⁹ CTCGCCCACCAGAAGTGG ¹⁶¹⁶
<i>psJCL</i> 12 26	sequencing	⁵⁸⁴ GATGGGCCTGAGGTAGTC ⁵⁶⁷

3. RESULTS

3.1. CAT

3.1.1. Purification of CAT

The purification procedure for CAT from isolated megagametophytes (7 DAI) is summarized in Table 3.1 and Figure 3.1. Although consisting of six main steps, including four chromatographic separations (Figure 3.2), the procedure was carried out in less than 2 d. Following $(\text{NH}_4)_2\text{SO}_4$ fractionation and chromatography on DE-52 cellulose and Superdex-200, initial CAT preparations consistently revealed two dominant protein bands of 59 kDa and 64 kDa on SDS-PAGE (Figure 3.1, lane D). Because of numerous reports identifying CAT as a frequent contaminant of ICL and MS preparations from many higher plants (Servettaz et al., 1973; Köller and Kindl, 1977; Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1986), both enzymes were also assayed throughout the CAT purification procedure. Despite very low activity due to suboptimal extraction conditions, MS co-purified with CAT during the first four steps of the purification. MS was then completely removed from CAT on HTP, with the former not binding to the matrix and eluting with the flow-through. At this stage, protein blots with cotton CAT and ICL antibodies identified loblolly pine CAT as a 59 kDa protein and ICL as a 64 kDa contaminant (data not shown). Having completely removed MS, HTP chromatography also provided the first step at which CAT could be partially resolved from ICL (Figure 3.3 A). Since just those fractions containing high CAT activity and low ICL activity were

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TABLE 3.1. Purification of CAT from 20 g (FW) of 7 DAI loblolly pine megagametophytes

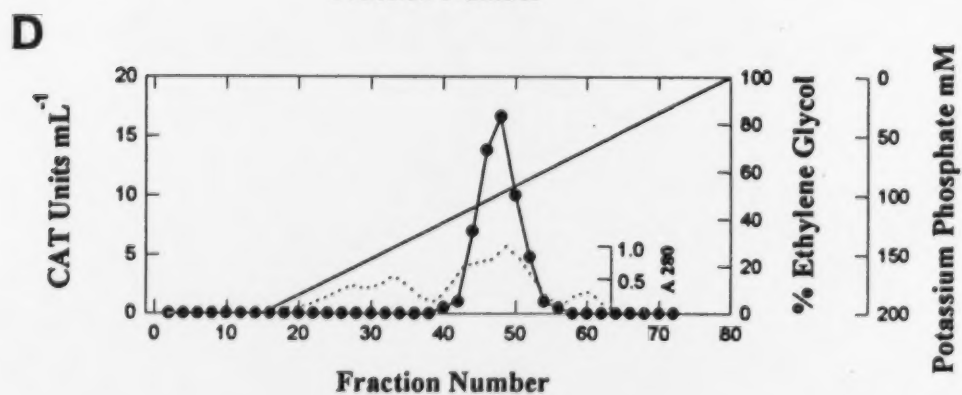
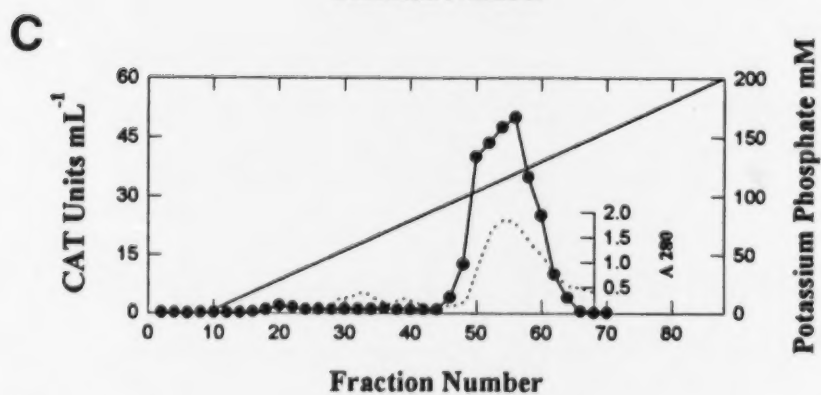
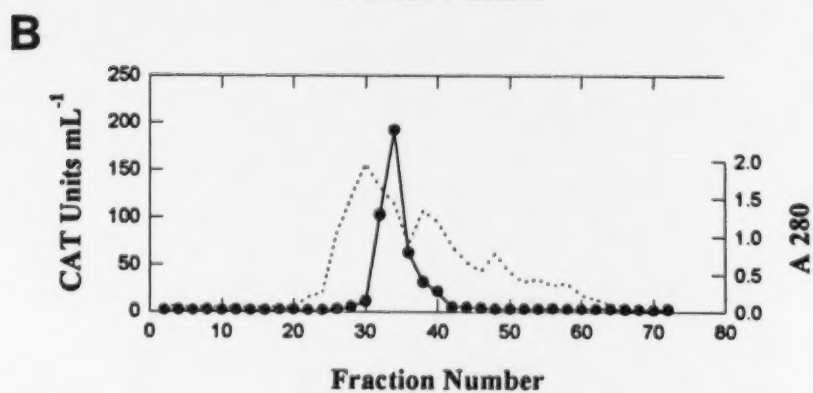
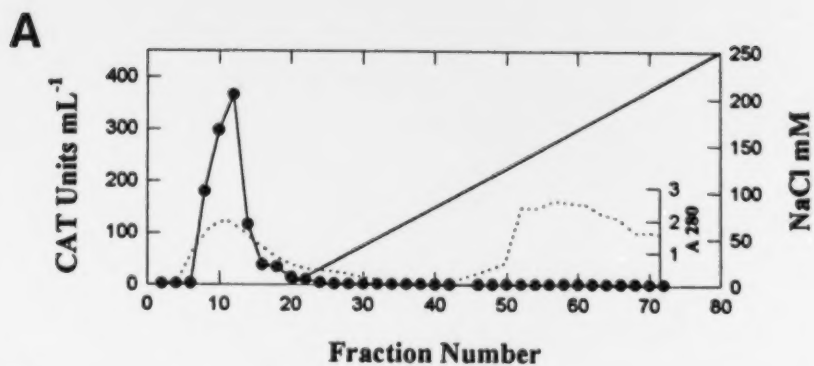
Purification Step	CAT Activity Total Units	Protein mg	Specific Activity Units mg ⁻¹	Yield %	Purification -fold
1. Acetone extract homogenate	6690	425.0	16	100	1.0
2. 30-70% satn. ammonium sulfate *	3106	83.4	37	46	2.3
3. DE-52 cellulose	1703	16.6	103	25	6.4
4. Superdex-200 *	1225	2.0	613	18	38.3
5. HTP	800	0.5	1600	12	100
6. Phenyl-Sepharose CL-4B	443	0.2	2215	7	138

* CAT activity assayed after (NH₄)₂SO₄ precipitation and overnight dialysis.



FIGURE 3.1. Progress of CAT purification from isolated megagametophytes 7 DAI. Pooled fractions from each stage of the purification procedure (Table I) were subjected to SDS-PAGE on 12% gels and visualized by Coomassie blue R staining. Lane A, acetone extract (crude homogenate); lane B, 30-70% saturation $(\text{NH}_4)_2\text{SO}_4$ fraction; lane C, pooled active fractions from DE-52 cellulose; lane D, pooled fractions from gel filtration on Superdex-200; lane E, pooled high CAT/ low ICL activity fractions from HTP; lane F, pooled purified CAT fractions from phenyl-Sepharose CL-4B; lane G, silver-stained pooled purified CAT (59 kDa) fractions from phenyl-Sepharose CL-4B. Fifteen μg of protein was applied to each lane, except in lanes F and G where 5 μg was loaded. Arrow indicates the relative mobility of CAT (59 kDa). Numerical values to the right of the gel are molecular masses (in kDa) of protein standards.

FIGURE 3.2. Purification of CAT. Elution profiles of CAT enzyme activity (●) and absorbance at 280 nm (.....) during (A) DE-52 cellulose anion-exchange, (B) Superdex-200 gel filtration, (C) HTP, and (D) phenyl-Sepharose CL-4B hydrophobic interaction chromatography. For all columns only peak CAT activity fractions were collected, pooled, and used for subsequent enzyme purification steps.



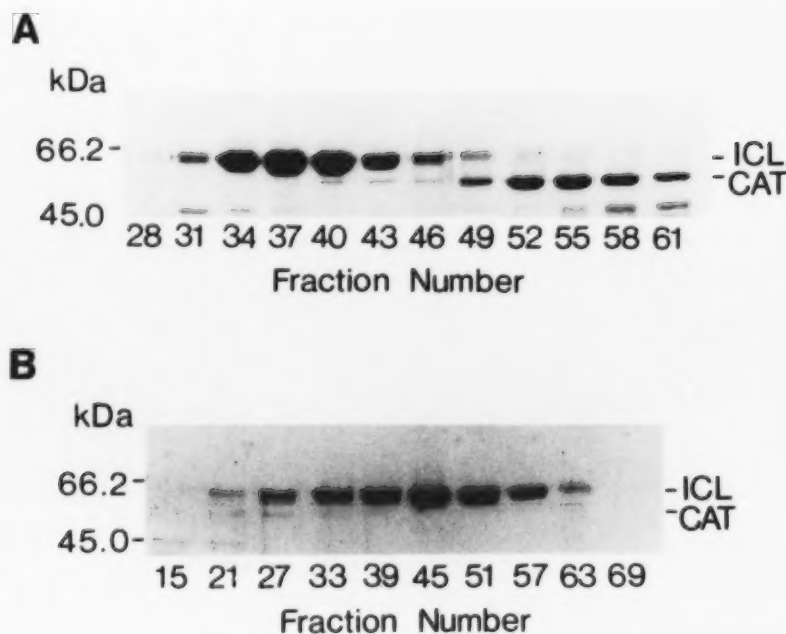


FIGURE 3.3. SDS-PAGE profiles of the purification and resolution of CAT and ICL A on HTP and octyl-Sepharose CL-4B column chromatography. Typical 12% SDS-PAGE Coomassie blue R stained gel profiles of eluted fractions from (A) HTP and (B) octyl-Sepharose CL-4B chromatography. Indicated to the right of the gel is the relative migration of purified CAT and ICL protein. Numerical values to the left of gels are the molecular masses (in kDa) of protein standards.

collected, only a 12% overall yield after HTP was achieved. However, this step still resulted in a substantial increase in CAT specific activity, purifying the protein about 2.6-fold (Table 3.1). The final separation CAT from ICL was accomplished by hydrophobic-interaction chromatography on phenyl-Sepharose CL-4B (Figure 3.3 B). Similar to previous findings, and in agreement with ICL's reportedly high hydrophobicity (Vanni et al., 1990), loblolly pine CAT did not bind as strongly to phenyl-Sepharose as did ICL, resulting in their complete resolution. CAT activity eluted from the column at approximately 45% (v/v) ethylene glycol/0.11 M potassium phosphate, and those active fractions with no detectable ICL activity were pooled, concentrated by ultrafiltration, and stored for antibody production. The final specific activity of the purified CAT preparation was about 2215 units mg^{-1} , representing an overall purification of about 140-fold (Table 3.1). Coomassie blue-stained (Figure 3.1, lane F) and silver-stained (Figure 1, lane G) SDS-PAGE of purified CAT revealed a principal band of 59 kDa. To further ascertain the purity of the final CAT preparation, no ICL enzyme activity or protein, as demonstrated by immunoblotting with ICL antibodies was detectable (data not shown).

3.1.2. Properties of CAT

As determined from its mobility during 12% SDS-PAGE (Figure 3.1), the subunit molecular mass of purified CAT is approximately 59 kDa. Gel filtration on a calibrated Sepharose CL-6B column indicated that the native molecular mass of CAT is 235,000 kDa (Figure 3.4). Apparently, and like nearly all CATs examined to date, loblolly pine CAT is a tetramer. Purified CAT did not react with Schiff's reagent (Fairbanks et al., 1971), indicating it is not a glycoprotein (data not shown). Similar to most known plant CATs (Eising and Gerhardt, 1986; Yamaguchi et al., 1986), the A_{208}/A_{405} ratio for the purified enzyme was 1.5 (data not shown). This value is consistent with 3-4 heme groups per enzyme molecule (Eising and Gerhardt, 1986).

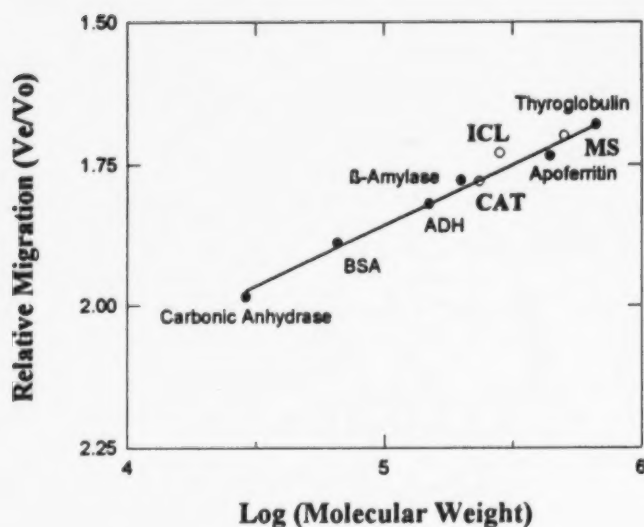


FIGURE 3.4. Native molecular weight determination of loblolly pine CAT, ICL, and MS by gel filtration on Sepharose CL-6B. Molecular weight markers (•) used for column standardization included; thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), ADH (150 kDa), and BSA (66 kDa). The relative elution of CAT, ICL and MS is indicated (○). All values shown are the mean of duplicate runs. Shown is a representative gel filtration profile standard curve for CAT with the molecular masses determinations of ICL and MS included.

3.1.3. Immunological Analysis

The monospecificity and fidelity of loblolly pine CAT antibodies was supported by the serum's ability to effectively immunotitrate all CAT activity from 7 DAI megagametophyte extracts (Figure 3.5). However, neither potential contaminants during the purification procedure (i.e. ICL and MS) were affected by the addition of CAT antiserum. Loblolly pine CAT antibodies cross-reacted with a single polypeptide on immunoblots of cell-free extracts of megagametophytes taken from germinated seeds of white spruce, lodgepole pine, and Douglas-fir, and two angiosperms, castor bean and cotton (Figure 3.6). The subunit molecular mass of cotton CAT was 57 kDa (Figure 3.6, lane B), whereas it was 59 kDa for all other species examined, including castor bean. To determine whether the subunit molecular mass of CAT from loblolly pine underwent any changes following mature seed imbibition, megagametophytes and seedlings were taken at various stages and immunoblots were probed with loblolly pine CAT antibodies (Figure 3.7). Cell-free extracts from megagametophytes isolated from mature seeds, stratified seeds, germinated seeds (7 DAI), and purified CAT fractions, all reacted with CAT antibodies, and a single, 59 kDa band appeared on all blots, indicating that at no time during stratification and post-germinative growth, as well as during purification, was the monomeric molecular mass of CAT altered. Loblolly pine glyoxysomal CAT subunit is the same size as that from cell-free extracts (59 kDa), indicating that no artefactual degradation of CAT by endogenous proteases during its extraction and purification occurs.

3.1.4. Multiple Isoforms

The presence of multiple native isoforms of CAT has been reported for a number of plant sources. To identify and characterize CAT isoforms in loblolly pine, cell-free extracts of megagametophytes following seed imbibition were subjected to non-denaturing IEF and stained for CAT enzyme activity. Shown in Figure 3.8 A is the CAT isoform pattern(s) from mature (lane A) and 12 DAI (lane B) megagametophytes subjected to IEF

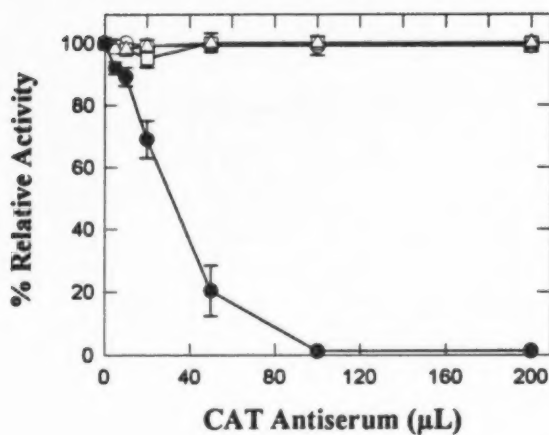


FIGURE 3.5. Immunotitration of CAT enzyme activity. 7 DAI isolated megagametophytes were extracted in 50 mM NaPO₄ pH 7.5, 0.1 mM leupeptin, microfuged, and supernatants diluted to contain equal amounts of CAT enzyme activity. Samples were then incubated at 25°C for 1 h, then overnight at 4°C, microfuged, and the supernatants assayed for CAT activity (●). When assayed for enzyme activity, CAT antiserum did not titrate any ICL (○) or MS (□), nor did control (preimmune serum) have any effect on CAT activity (Δ). Each value shown is the mean of three determinations ±SD.

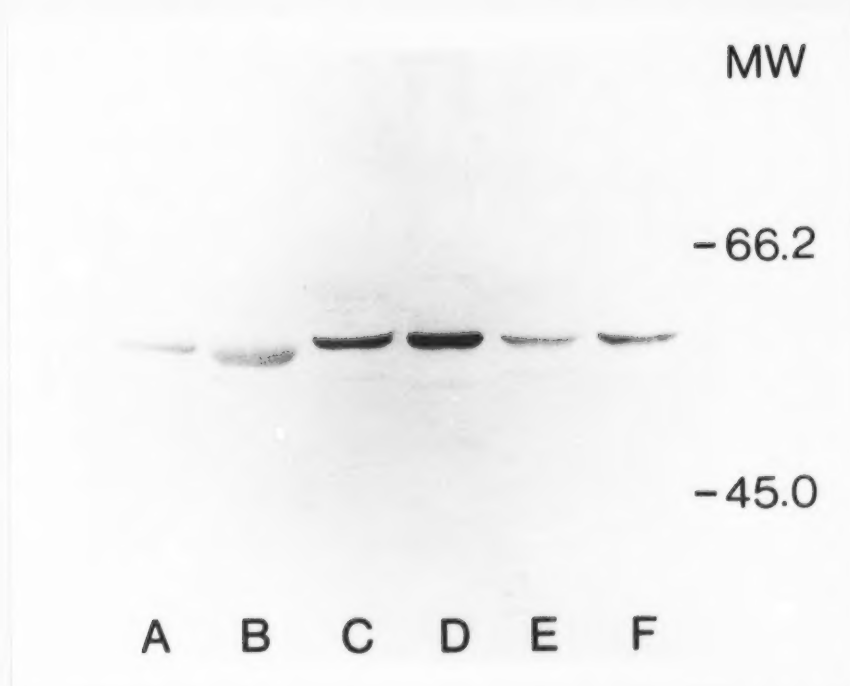


FIGURE 3.6. Immunoblot comparison of CAT from various gymnosperm and angiosperm seeds. Cell-free extracts from castor bean endosperm (4 DAI) (lane A), cotton cotyledons (4 DAI) (lane B), and megagametophytes isolated from seeds 7 DAI of Douglas fir (lane C), loblolly pine (lane D), lodgepole pine (lane E), and white spruce (lane F) were separated on SDS-PAGE, electroblotted to nitrocellulose, and probed with loblolly pine CAT antiserum. All lanes contained 20 μ g of protein. Numerical values to the right of the blot are the molecular masses (in kDa) of protein standards.

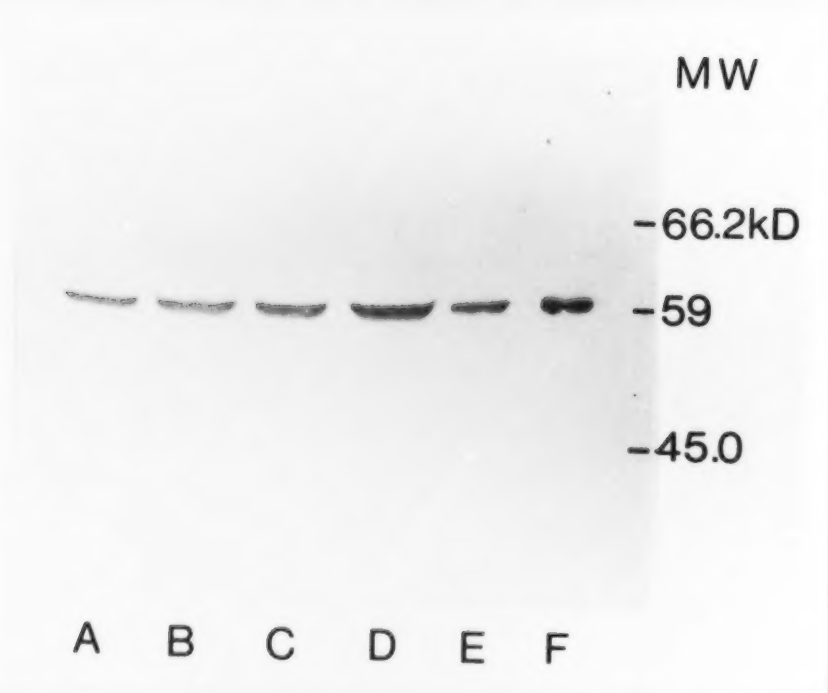


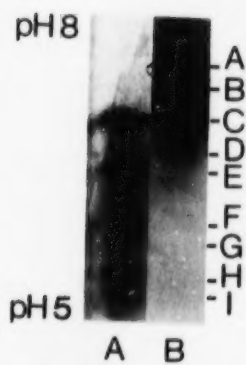
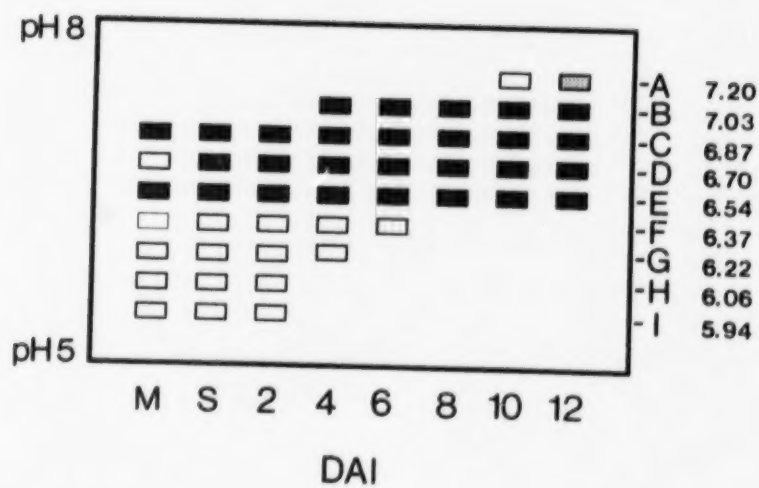
FIGURE 3.7. Immunoblot comparison of loblolly pine CAT. Protein cell-free extracts from 7 DAI seedling cotyledons (lane A), mature seed megagametophytes (lane B), stratified seed megagametophytes (lane C), 7 DAI megagametophytes (lane D), purified CAT protein from phenyl-Sepharose (lane E), and glyoxysomes isolated from megagametophytes 7 DAI (lane F). Protein (15 μ g) was applied to each lane, except in lane F where 5 μ g was loaded. Numerical values to the right of the blot are molecular masses (in kDa) of protein standards.

and stained for CAT activity. Because of their low activity, a number of the CAT isoforms (i.e. A, and F-I) were not resolved when photographed, and were only distinguishable on the original gels. Therefore, Figure 3.8 B is a representative zymogram of the CAT isoform patterns in megagametophytes following seed imbibition. Overall at least nine native CAT isoforms (A-I), including one dominant isoform in all stages (isoform C, pI 6.87), were identified in the megagametophyte following seed imbibition. During seed stratification, germination, and post-germinative growth, the patterns of CAT isoforms in the megagametophyte underwent characteristic changes. In general, the acidic charged CAT isoforms found in the megagametophyte of the mature seed (isoforms F-I) were replaced by the accumulation of more basic forms (isoforms A and B) (Figure 3.8 B).

Because of a number of precautions outlined by other laboratories interested in CAT heterogeneity, the electrophoretic pattern of loblolly pine CAT isoforms was determined not to be the result of enzyme aging, limited proteolysis, or experimental manipulation(s) (data not shown) (Moridoffer-Zwez et al., 1969; Holmes and Masters, 1972; Kunce and Trelease, 1986). The consistent resolution of castor bean and cotton native CAT into their published isoelectric isoform families (Figure 3.9 A and B) (Kunce and Trelease, 1986; Mullen and Gifford, 1995), using our experimental procedures, provided the strongest evidence that the loblolly pine CAT multiplicity reported here is not the result of experimental conditions.

The native CAT isoforms that were difficult to resolve on IEF gels stained for CAT enzyme activity were detectable immunologically. In Figure 3.10 is a representative protein blot of megagametophyte protein extracts probed with CAT antiserum following IEF. Again, and similar to the isoform activity pattern shown in Figure 3.8, nine native CAT isoforms, including the dominant isoform C, were identified. However, there were a number of discrepancies between the CAT enzyme activity and protein blot patterns. In blots, CAT isoforms A and B were observed at all stages examined, though they were undetectable in the megagametophyte from mature and stratified seed after activity staining. Similarly, CAT isoforms F through I were undetectable with activity

FIGURE 3.8. Non-denaturing IEF electrophoretic patterns and representative zymogram of megagametophyte native CAT isoforms. (A). Mature (lane A) and 10 DAI (lane B) isolated megagametophyte cell-free extracts were subjected to non-denaturing IEF (pH 5-8) at 500 V for 15 min, 750 V for 2 h, and stained for CAT enzyme activity with DAB. All samples contained 20 mM DTT and were electrophoresed immediately after extraction. Indicated to the right of the gels are the individual CAT isoforms (A-I). Because of printing some isoforms were observed only on the original gels. (B). Native CAT isoform patterns in the megagametophyte from mature (M), stratified (S), and seeds from various stages after imbibition at 30°C. Again, because of their poor resolution after photography, CAT isoforms are presented here as a zymogram. Shown for each stage is the CAT isoform pattern present and their individual relative activity staining intensity (□ faint → ■ intense). Acidic isoforms F-I gradually disappear during germination and into post-germinative growth, whereas isoforms A and B appear during post-germination. Indicated to the right of the zymogram is each CAT isoform (A-I) and their pI's.

A**B**

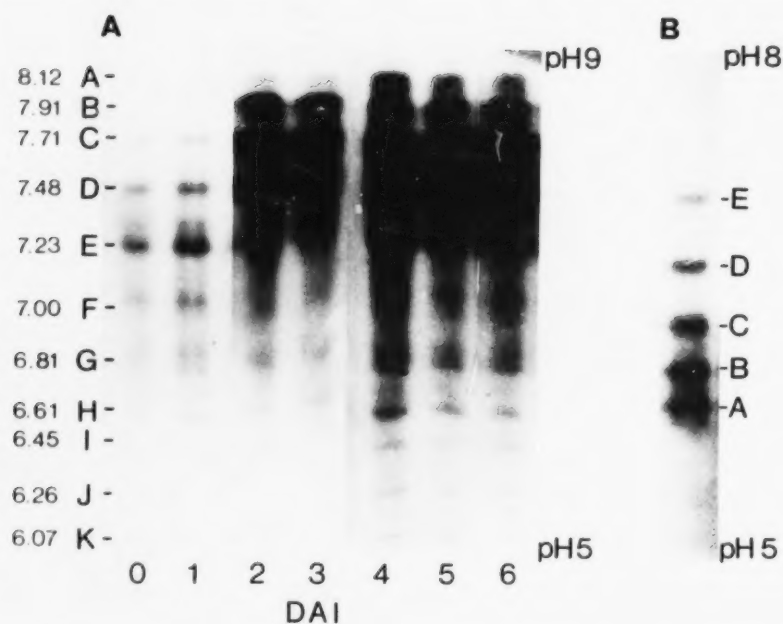


FIGURE 3.9. Castor bean and cotton isoelectric CAT isoform patterns following non-denaturing IEF. Cell-free extracts from (A) castor bean endosperms of seeds imbibed for 0-6 days and (B) dark-grown 4 DAI cotton cotyledons were loaded at the anode end of a IEF gel, electrophoresed for 15 min at 500 V, 750 V for 2 h, then stained for CAT enzyme activity with DAB. All samples contained equal amounts of protein (5 μ g), 0.1 mM leupeptin, 20 mM DTT, and were immediately electrophoresed after extraction. The pI for each castor bean isoform (A-K) is indicated to the left of the gel (A) (Mullen and Gifford, 1995). CAT isoforms from cotton cotyledons (A-E) are indicated to the right of the gel (B) and are those described by Kunc and Trelease (1986).

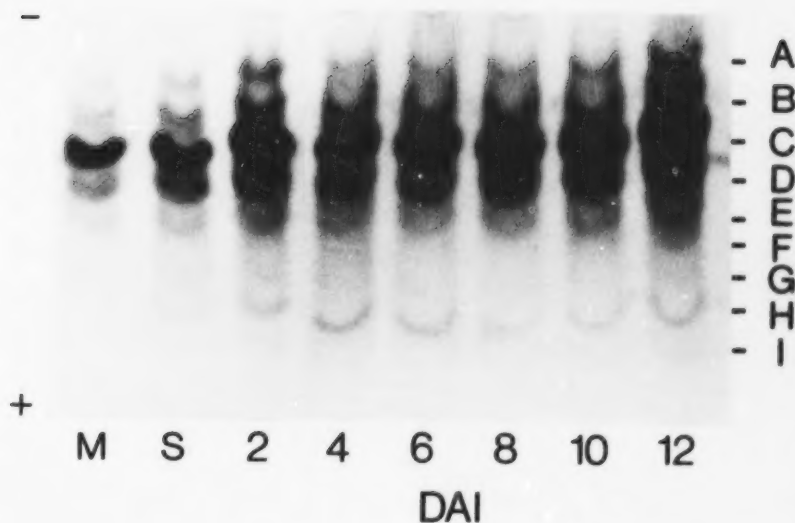


FIGURE 3.10. Immunoblot of loblolly pine native CAT isoforms after non-denaturing IEF. Megagametophyte cell-free extracts from mature (M), stratified (S), and seeds taken at various stages after imbibition at 30°C were subjected to non-denaturing IEF, electroblotted to nitrocellulose, and probed with CAT antiserum. For all lanes, equal amounts of CAT enzyme activity (5 units) were applied. Indicated to the right of the blot are the CAT isoforms A-I. Indicated to the left of the blot are the anode (+, pH 5) and cathode (-, pH 8) ends of the gel.

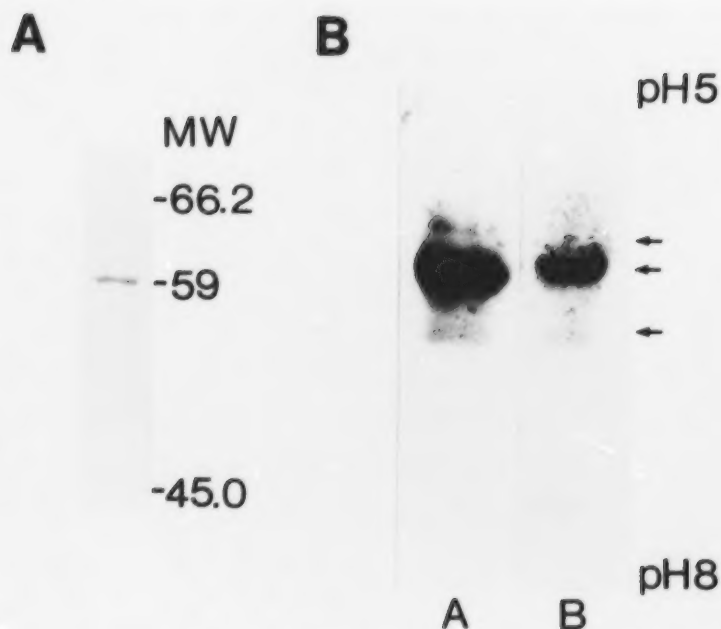


FIGURE 3.11. Immunoblot of CAT protein subunits on 7% SDS-PAGE and denaturing urea IEF. (A). 8 DAI megagametophyte cell-free extracts were subject to 7% SDS-PAGE, electroblotted to nitrocellulose, and probed with CAT antiserum. Indicated to the right of the gel is the molecular masses (in kDa) of protein standards, and the relative migration of CAT as determined using purified protein as a standard. (B). 8 DAI megagametophyte cell-free extracts (lane A) and purified CAT protein (lane B) were subjected to denaturing urea IEF (pH 5-8), electroblotted to nitrocellulose, and probed with CAT antiserum. Samples were loaded at the acidic end of the gel and electrophoresed at 4°C at 200 V for 2.5 h and 400 V for an additional 30 min. Following protein blotting and probing with CAT antiserum three charged isoforms (indicated to the right of the blot →) were detected.

staining during the later stages of post-germinative growth, though they were clearly visible on blots. Overall, on blots, all nine CAT isoforms were present during every developmental stage examined, and their relative accumulation remained unchanged during stratification, germination, and post-germinative growth (Figure 3.10).

In germinated castor bean endosperm, 7% SDS-PAGE was used to demonstrate that CAT heterogeneity involves at least two CAT subunits with molecular masses of 57 and 59 kDa (Mullen and Gifford, 1995). To begin to examine the subunit composition of the nine megagametophyte native CAT isoforms, protein extracts from 8 DAI isolated megagametophytes were subjected to 7% SDS-PAGE/protein blotting and probed with CAT antiserum (Figure 3.11 A). Electrophoresis on 7 % SDS-PAGE, as opposed to 12 % SDS-PAGE (Figure 3.1), was used in hopes of further resolving, or separating the 59 kDa CAT species into more than one polypeptide. However, only one CAT subunit polypeptide (59 kDa) was observed (Figure 3.11 A). This suggested that loblolly pine CAT is not composed of protein subunits which differed in molecular mass. To determine if this 59 kDa CAT species was instead composed of polypeptides which differ on the basis of charge, 8 DAI megagametophyte cell-free extracts (lane A) and purified CAT protein (lane B) were subjected to denaturing urea IEF, blotted onto nitrocellulose, and probed with CAT antiserum (Figure 3.11 B). When loaded at the acid end of the gel and focused toward the cathode to prevent alkaline aggregation (Ni et al., 1990), at least three separate charged CAT subunit isoforms were resolved (Figure 3.11 B). These results suggest that loblolly pine CAT is composed of at least three protein subunits that differ on the basis of charge and likely undergo combination and recombination associations (Ni and Trelease, 1991), or post-translational modifications (Eising et al., 1990) to form the nine native CAT isoforms.

3.2. ICL

3.2.1. Purification of ICL

The overall purification of ICL from isolated 9 DAI megagametophytes is summarized in Table 3.2 and Figure 3.12. During ICL purification, chromatography on DE-52 cellulose consistently resolved two separate peaks of ICL activity (ICL A and ICL B) (Figure 3.13 A). The elution profile of ICL activity from the column did not alter when either PMSF (1 mM), leupeptin (1 μ M), benzamidine (2 mM), and/or EDTA was included (data not shown). ICL A always eluted with the flow through fractions and represented the dominant active form. ICL B remained bound to the column until eluted with approximately 100 mM NaCl. To determine whether both forms were related, DE-52 effluent fractions were analyzed by SDS-PAGE, and protein blotting. When compared to the migration of purified ICL A, Coomassie blue stained SDS-PAGE analysis of peak ICL B fractions, revealed a similar dominant band migrating at approximately 64 kDa (Figure 3.14 A). However, protein blots of ICL B (fractions #35-44) probed with ICL A antiserum indicated that the enzyme was not antigenically cross-reactive (Figure 3.14 B). Even when peak ICL B fractions were overloaded and/or ICL A antiserum was used only as a 1:100 dilution, rather than 1:5000 as performed in all other experiments, ICL B was immunologically undetectable (data not shown).

Following the elution of flow through fractions from DE-52 cellulose, ICL A fractions were collected for further purification. After ammonium sulfate concentration and buffer exchange, pooled ICL A fractions were loaded directly onto a Sepharose CL-6B gel filtration column. ICL A activity eluted from this column as a single peak (Figure 3.13 B). Fractions from this peak were collected, dialyzed against buffer C overnight, and loaded onto an HTP column. HTP chromatography was the first step in the isolation procedure that separated ICL A from CAT, a frequent contaminant of other ICL plant preparations (Figure 3.13 C; see Figure 3.3 A) (Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1986). Only those HTP fractions high in ICL A and low in CAT specific activity were collected, ammonium sulfate precipitated, and resuspended in buffer

TABLE 3.2. Purification of ICL from 20 g (FW) of 9 DAI loblolly pine megagametophytes

Purification Step	ICL Activity Total Units	Protein mg	Specific Activity Units mg ⁻¹	Yield %	Purification -fold
1. Acetone extract	30.9	351.1	0.088	100	1.0
homogenate					
2. 0-50% satn. ammonium sulfate *	25.8	47.6	0.542	83	6.2
3. DE-52 cellulose	20.3	14.5	1.400	66	15.9
4. Sepharose CL-6B *	17.5	7.3	2.397	57	27.2
5. HTP	11.5	3.2	3.593	37	40.8
6. Octyl-Sepharose CL-4B	9.2	2.1	4.455	30	50.6

* ICL activity assayed after (NH₄)₂SO₄ precipitation and overnight dialysis.

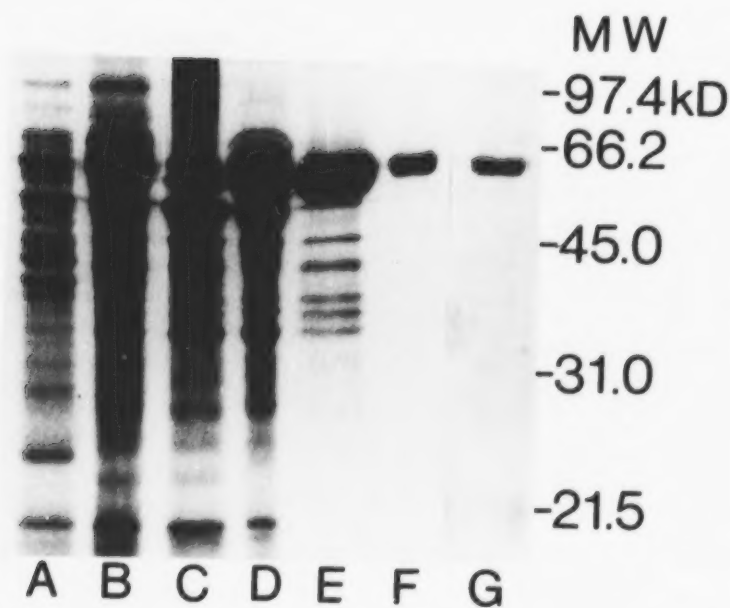
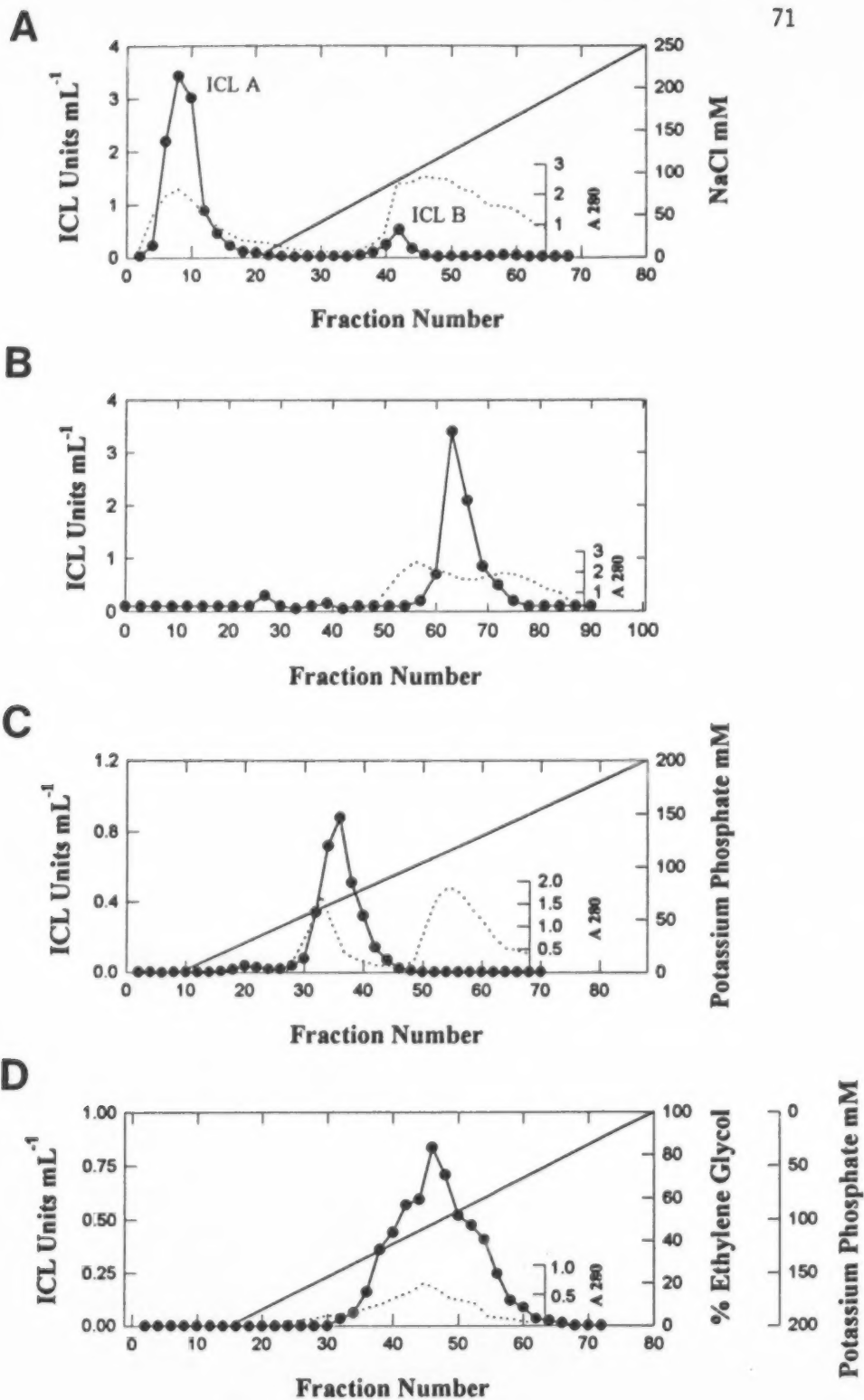


FIGURE 3.12. Progress of ICL A purification from 9 DAI megagametophytes. Pooled fractions from each of the purification steps were subjected to 12% SDS-PAGE and visualized by either Coomassie blue R (lanes A-F) or silver stained (lane G). The total amount of protein loaded for each lane is indicated in parentheses. A, acetone extract (crude homogenate) (10 μ g); B, 0-50% (saturation) $(\text{NH}_4)_2\text{SO}_4$ resuspended pellet (10 μ g); C, pooled ICL A active fractions from DE-52 cellulose (10 μ g); D, Sepharose CL-6B gel filtration pooled active fractions (10 μ g); E, HTP pooled active fractions (7.9 μ g); F, octyl-Sepharose CL-4B pooled active fractions (2.9 μ g); and G, silver stained pooled active fractions from octyl-Sepharose CL-4B (2.9 μ g). Numerical values to the right of the gel are the molecular masses (in kDa) of protein standards.

FIGURE 3.13. Purification of ICL A. Elution profiles of ICL enzyme activity (•) and absorbance at 280 nm (—) during (A) DE-52 cellulose anion-exchange, (B) Sepharose CL-6B gel filtration, (C) HTP, and (D) octyl-Sepharose CL-4B hydrophobic interaction chromatography. For all columns only peak ICL activity fractions were collected, pooled, and used for subsequent enzyme purification steps.



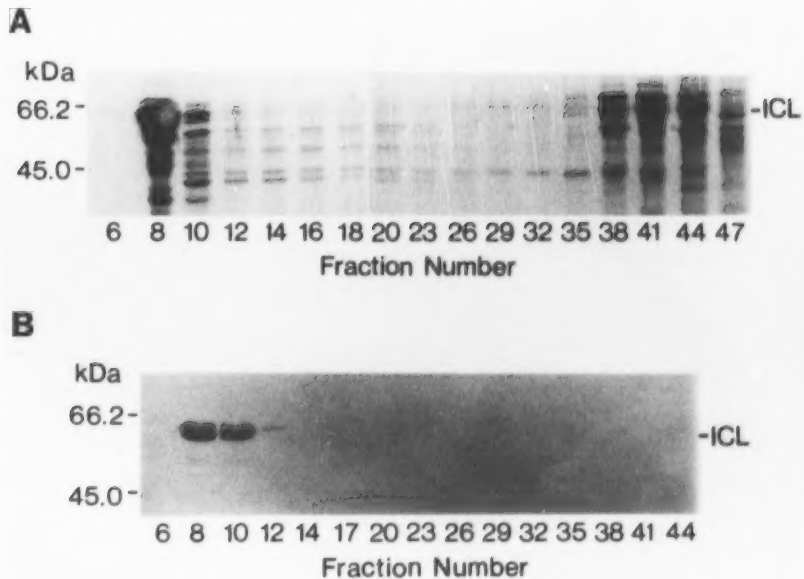


FIGURE 3.14. SDS-PAGE and immunoblot profile of ICL A and ICL B on DE-52 cellulose. (A). Coomassie blue R stained 12% SDS-PAGE analysis of DE-52 cellulose fractions. Indicated to the right of the gel is the relative migration of ICL as determined by using purified protein as a standard. (B). Protein blot analysis of DE-52 cellulose fractions probed with ICL A antiserum. DE-52 cellulose fractions were subjected to 12% SDS-PAGE as above in (A), electroblotted to nitrocellulose and probed with ICL A antiserum. Numerical values to the left of the gel (A) or protein blot (B) are the molecular masses (in kDa) of protein standards.

D. The complete resolution and subsequent final purification of ICL A from CAT involved a high-resolution octyl-Sepharose CL-4B hydrophobic interaction column (Figure 3.13 D; see Figure 3.3 B). CAT eluted quickly from the column whereas ICL A was retained until its elution at approximately 50% ethylene glycol/100 mM potassium phosphate. Pooled ICL A active fractions from this step were concentrated and stored for antibody production. Purified ICL A fractions from octyl-Sepharose CL-4B revealed no contaminating proteins when run on 12% SDS-PAGE and stained with either Coomassie blue or silver (Figure 3.12, lanes F and G). The final specific activity of purified ICL A was 4.45 Units mg^{-1} protein which represented a 50.6-fold purification (Table 3.2). Yield of the purified enzyme was 30% of the original homogenate activity, a value that is comparably higher than those previously reported for other plant ICL preparations (Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1986).

3.2.2. Multiple Subunit Isoforms

Following its initial separation from ICL B, and its final resolution from CAT on octyl-Sepharose CL-4B, purified loblolly pine ICL A was analyzed on 7% SDS-PAGE. A Coomassie stained profile of purified ICL A revealed at least two distinct subunits (Figure 3.15, lane B). ICL A 1 and ICL A 2 had molecular masses of 64 and 63.5 kDa, respectively. Both forms were antigenically cross-reactive when probed with ICL A antiserum (Figure 3.15, lanes C and D), with ICL A 2 representing the dominant form. Furthermore, both ICL A 1 and ICL A 2 were detectable in isolated megagametophyte glyoxysomal fractions probed with ICL A antiserum (Figure 3.15, lane D).

To further examine the multiplicity of ICL A subunit isoforms, purified ICL A protein extract was subjected to IEF/SDS-PAGE and protein blotting. When separated on 7% SDS-PAGE in the second dimension, blotted onto nitrocellulose, and probed with ICL A antiserum at least three isoforms of ICL subunits could be detected (Figure 3.16). ICL A 1 (64 kDa) focused at a pI of around 7.5. ICL A 2 (63.5 kDa) separated into two charged

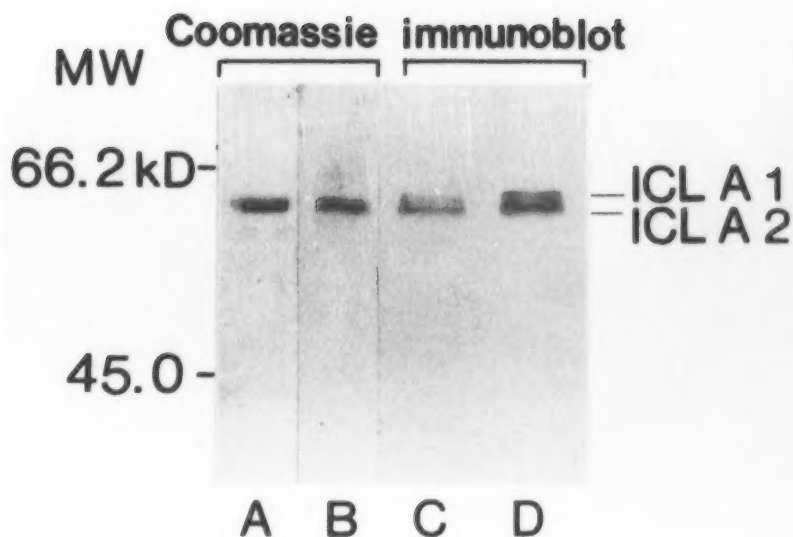


FIGURE 3.15. SDS-PAGE and immunoblot analysis of ICL A 1 and ICL A 2 subunits. Purified ICL A was subjected to either 12% (lane A) or 7 % (lane B) SDS-PAGE and stained with Coomassie blue R, or purified ICL A fractions and isolated glyoxysomal proteins were analyzed on 7% SDS-PAGE, electroblotted to nitrocellulose and probed with ICL A antiserum (lanes C and D). Both ICL A 1 (64 kDa) and ICL A 2 (63.5 kDa) subunits were immunologically cross-reactive when probed with ICL A antiserum and each was identified in the sucrose density-purified glyoxysomal fractions (lane D). Indicated to the left of the figure are the molecular masses (in kDa) of protein standards analyzed on a 7% SDS-PAGE gel.

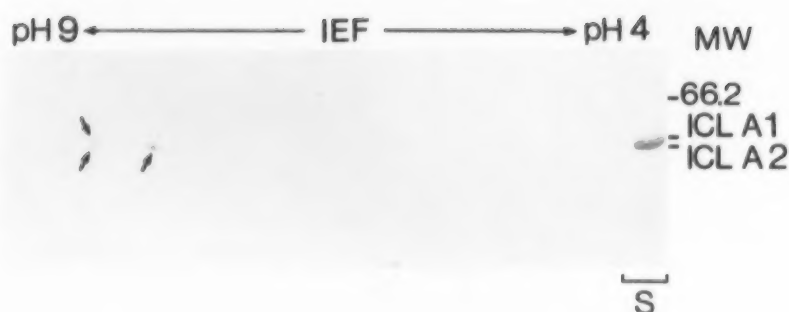


FIGURE 3.16. Immunoblot analysis of ICL A protein subunits after IEF/SDS-PAGE. Purified ICL A protein was subjected to IEF and 7% SDS-PAGE, electroblotted to nitrocellulose, and probed with ICL A antiserum. Immunologically detectable polypeptides representing individual charged ICL A isoforms are indicated (→). In lane S is the same purified ICL protein sample subjected to second dimension 7% SDS-PAGE only. Indicated to the right of the blot is the relative migration of ICL A 1 and ICL A 2 as determined by staining an identical gel with Coomassie blue R. Numerical values to the right of the blot are the molecular masses (in kDa) of protein standards. Indicated at the top of the blot is the pH range (pH 4-9) of first dimension IEF.

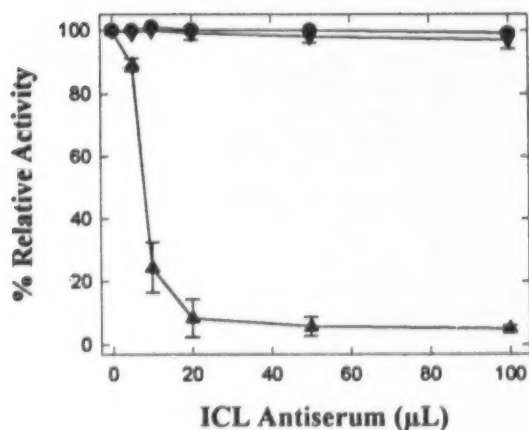


FIGURE 3.17. Immunotitration of ICL enzyme activity. 9 DAI isolated megagametophytes were extracted in 50 mM NaPO₄ pH 7.5, 0.1 mM leupeptin, microfuged, and supernatants diluted to contain equal amounts of ICL enzyme activity. Samples were then incubated at 25°C for 1 h, then overnight at 4°C, microfuged, and the supernatants assayed for ICL (▲) or CAT (●) enzyme activity. For controls, preimmune serum did not titrate any ICL activity (Δ). Each value shown is the mean of three determinations $\pm$ SD.

isoforms; one with the same pI as ICL A 1, and another form which was slightly more acidic. Interestingly, these same two charged isoforms of ICL A 2 were not resolved during standard SDS-PAGE/protein blotting experiments (lane S) (Figure 3.16).

3.2.3. Immunological Characterization

To demonstrate that antibodies to loblolly pine ICL A were monospecific, a number of immunological experiments were carried out. Immunotitrations of ICL enzyme activity showed that the addition of only 20 μ l of ICL A antiserum eliminated nearly all of the ICL activity in cell-free extracts of 9 DAI megagametophytes (Figure 3.17). Residual ICL activity is likely due to the ICL B form which is immunologically distinct from ICL A. Controls, including the addition of preimmune serum, or assays for a reduction of CAT activity with the inclusion of ICL A antiserum, did not result in any precipitation of ICL, or CAT activity, respectively (Figure 3.17). This latter observation is particularly important because of the number of steps required and the difficulty we observed in purifying ICL from contaminating CAT.

The fidelity and monospecificity of the polyclonal antibodies was also evaluated by immunoprecipitation. Isolated 9 DAI megagametophytes were labelled for 3 h with [^{35} S]-methionine and buffer-soluble proteins were extracted. When these labelled cell-free extracts were immunoselected with ICL A antiserum only one polypeptide migrating to the same position as purified ICL was detectable after 12% SDS-PAGE fluorography (Figure 3.18, lane B). Analysis on 7% gels resolved two polypeptides with molecular masses of 64 and 62.5 kDa, indicating that both ICL A 1 and ICL A 2 were immunoselected and are actively synthesized *de novo* in 9 DAI megagametophytes (data not shown). The effectiveness of our purification procedure for the elimination of contaminating CAT was further supported by the observation that no CAT polypeptide (59 kDa) was detectable on fluorographs. Incubation of *in vivo* labelled proteins with preimmune serum as a control resulted in no radioactive immunoprecipitates detected on

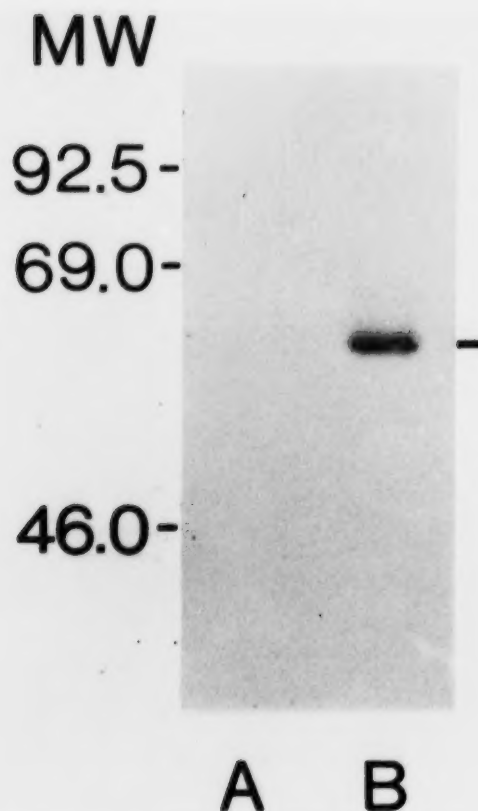


FIGURE 3.18. SDS-PAGE and fluorography of *in vivo* synthesized ICL A immunoselected from 9 DAI [^{35}S]-methionine labelled megagametophytes. Nine DAI megagametophytes were labelled for 3 h with [^{35}S]-methionine and buffer-soluble proteins extracted. ICL A was immunoselected with preimmune (lane A) or ICL A antiserum (lane B) and subjected to 12% SDS-PAGE and fluorography. No ICL A protein was detectable in lane A. Indicated to the right of the fluorograph is the relative migration of purified ICL A protein on an identical Coomassie blue R stained gel. Numerical values to the left of the gel are the molecular masses (in kDa) of protein standards.

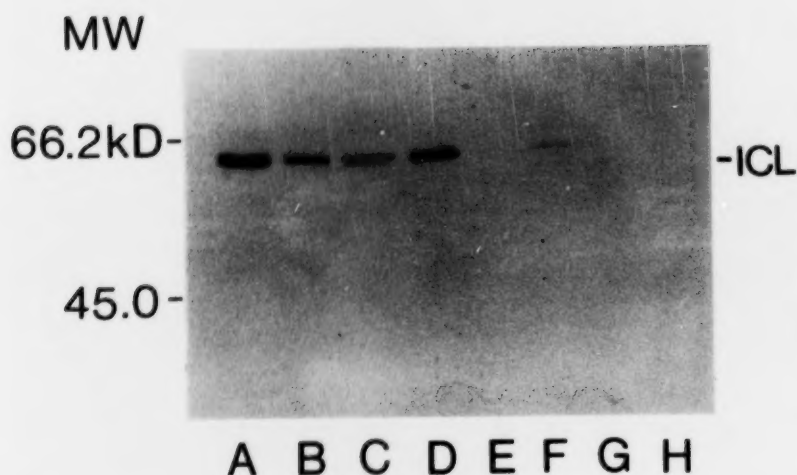


FIGURE 3.19. Immunoblot comparison of loblolly pine ICL. Various loblolly pine cell-free extracts were subjected to 12% SDS-PAGE, electroblotted to nitrocellulose, and probed with ICL A antiserum. Protein extracts examined included; purified ICL A protein fractions (lane A), glyoxysomes isolated from 9 DAI megagametophytes (lane B), mature seed megagametophytes (lane C), 9 DAI megagametophytes (lane D), mature seed embryos (lane E), 9 DAI embryos (lane F), 4 month seedling roots (lane G), and 4 month seedling needles (lane H). Identical protein (20 μ g) was loaded for each lane except in lane A where 1.4 μ g was loaded. Numerical values to the left of the blot are molecular masses (in kDa) of protein standards.

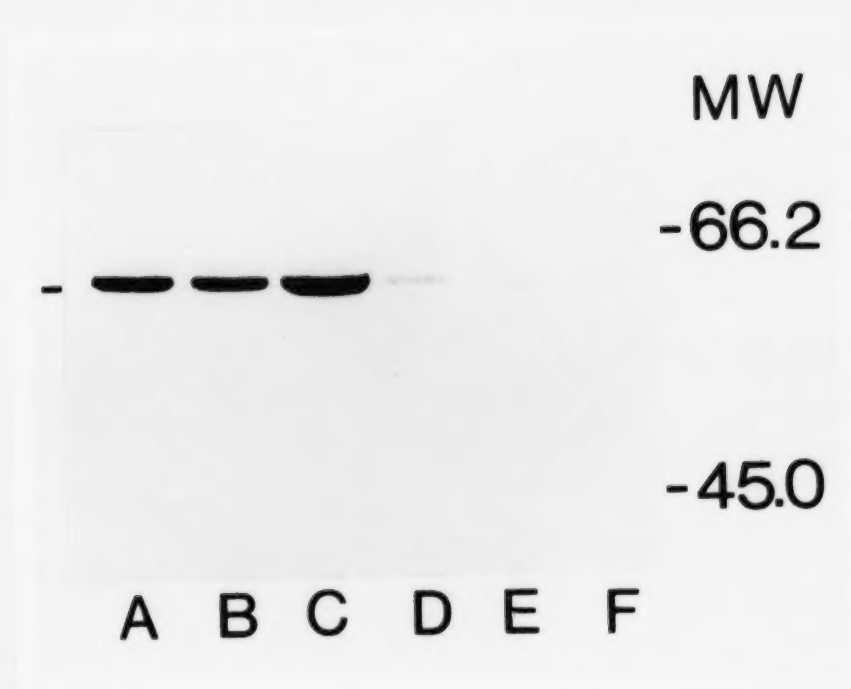


FIGURE 3.20. Immunoblot comparison of ICL from various gymnosperm and angiosperm seeds. Cell-free extracts from megagametophytes isolated from seeds 9 DAI from lodgepole pine (lane A), white spruce (lane B), loblolly pine (lane C), Douglas fir (lane D) and cotton cotyledons (4 DAI) (lane E) and castor bean endosperm (4 DAI) (lane F) were subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with loblolly pine ICL A antiserum. All lanes contained 20 μ g of protein. Numerical values to the right of the blot are the molecular masses (in kDa) of protein standards.

fluorographs (Figure 3.18, lane A).

Protein blots of purified ICL A and cell-free extracts from isolated glyoxysomes, as well as a number of loblolly pine developmental- and tissue specific extracts were probed with ICL A antiserum (Figure 3.19). Besides supporting the monospecificity of our polyclonal antibodies, these data also indicated that at no time during purification or post-germinative growth was the monomeric molecular mass of ICL A altered. In all cases where ICL protein was present or at detectable levels, a similar immunoreactive polypeptide appeared on blots. As previously demonstrated in Figure 3.15, immunoreactive ICL for all lanes shown in Figure 3.19, including 9 DAI embryo, migrated as two bands when analyzed on 7% SDS-PAGE (data not shown). ICL protein was not detectable in cell-free extracts of isolated loblolly pine needles or roots (Figure 3.19, lanes G and H). Protein blot analysis of 9 DAI embryo cell-free extracts indicated that, although at much lower levels than at comparable megagametophyte stages, apparently the same immunoreactive polypeptide as purified megagametophyte ICL A was detectable (Figure 3.19, lane F). ICL protein was never detected in mature embryos (Figure 3.19, lane E) even when gels were overloaded (data not shown). In isolated megagametophyte cell-free extracts, ICL protein was detectable at very low levels in the mature seed (lane C), then at considerably higher levels at 9 DAI (lane D).

Figure 3.20 shows a 12% SDS-PAGE/protein immunoblot of total buffer-soluble protein extracts from a number of conifer and non-coniferous seeds probed with loblolly pine ICL A antiserum. ICL A antiserum cross-reacted with a polypeptide of 64 kDa from germinated seeds of lodgepole pine (lane A), white spruce (lane B), loblolly pine (lane C), Douglas-fir (lane D), cotton (lane E), and castor bean (lane F) (Figure 3.20). However, the serological cross-reactivity of loblolly pine ICL antiserum was higher for members of *Pinaceae* (lanes A-D) as compared to the two angiosperm species examined (lanes E and F).

3.3. MS

3.3.1. Purification of MS

Following a modification of the purification procedure described by Trelease et al. (1987), MS was purified to apparent homogeneity from megagametophytes isolated from seeds 10 DAI. An overview of the isolation of MS is summarized in Table 3.3 and Figure 3.21. Following 0-35% saturation ammonium sulfate fractionation, and by the precautionary inclusion of sodium glyoxylate in all buffers thereafter, loblolly pine MS eluted from the Sepharose CL-6B column as a single peak (Figure 3.22 A). Inclusion of sodium glyoxylate during MS purification procedures has been previously demonstrated to prevent enzyme aggregation into higher molecular mass species (Köller and Kindl, 1977). Loblolly pine MS did not bind to DE-52 cellulose ion exchange, phenyl-Sepharose CL-4B hydrophobic interaction columns, or HTP (Figure 3.22 B, C and D). Chromatography on the phenyl-Sepharose CL-4B was responsible for the complete resolution of CAT (59 kDa) and ICL (64 and 63.5 kDa) from MS (Figure 3.21, lane E), the former being previously reported as contaminants of other MS preparations (Servettaz et al., 1973; Köller and Kindl, 1977). The final purification of loblolly pine MS on HTP resulted in an almost 145-fold enrichment of the specific activity up to 308 units mg^{-1} (Table 3.3)

3.3.2. Properties of MS

Purified loblolly pine MS showed a single polypeptide band of 62 kDa when run on SDS-PAGE and stained with either Coomassie blue R or silver (Figure 3.21, lanes F and G). The native molecular mass of the enzyme as estimated by standardized gel filtration of the final preparation was found to be approximately 520 kDa (Figure 3.4).

When protein blots of loblolly pine cell-free extracts were probed with serum from rabbits elicited with purified MS protein, some immunostaining of polypeptides other than the 62 kDa MS was observed (Figure 3.23, lane A). Included in this group of cross-

TABLE 3.3. Purification of MS from 15 g (FW) of 10 DAI loblolly pine megagametophytes

TABLE 3.3. Purification of MS from 15 g (FW) of 10 DAI loblolly pine megagametophytes						
Purification Step	MS Activity	Protein	Specific Activity	Yield	Purification	
	Total Units	mg	Units mg ⁻¹	%	-fold	
1. Acetone extract	1061	495.9	2.14	100	1.0	
homogenate						
2. 0-35% satn. ammonium sulfate	397	41.3	9.61	37	4.5	
3. Sepharose CL-6B	209	16.1	13.0	20	6.1	
4. DE-52 cellulose	161	9.52	16.9	15	7.9	
5. Phenyl-Sepharose CL-4B	64.5	0.32	202	6.1	94	
6. HTP	52.4	0.17	308	4.9	144	

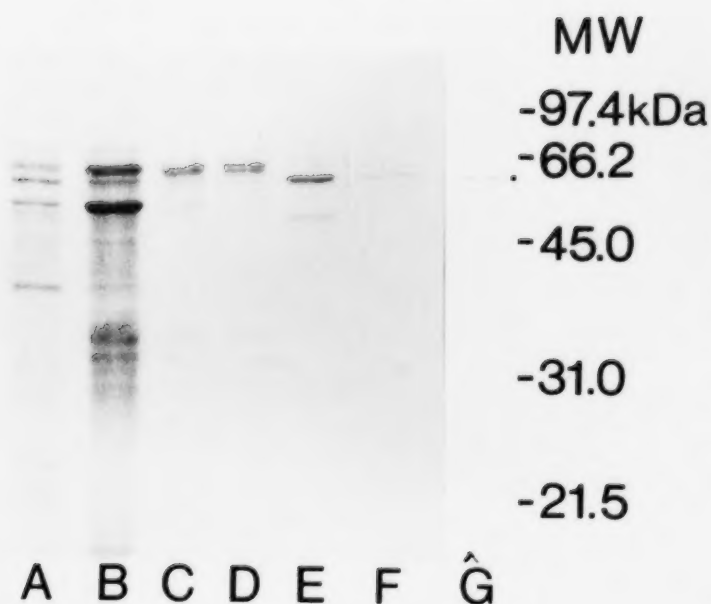
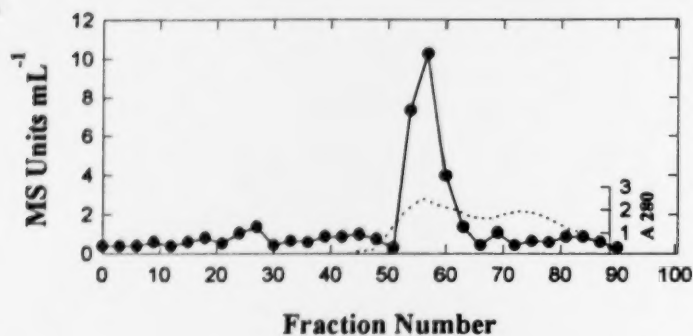
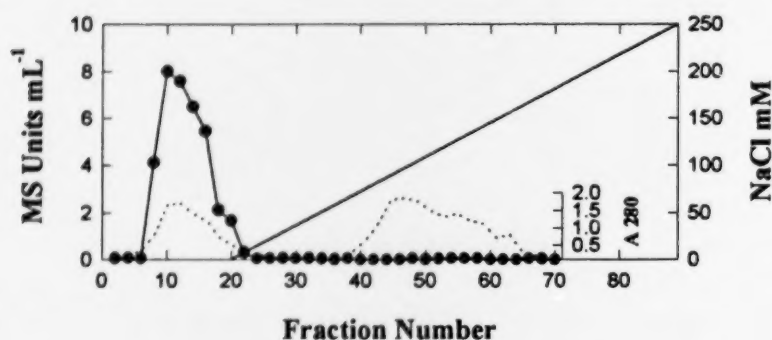
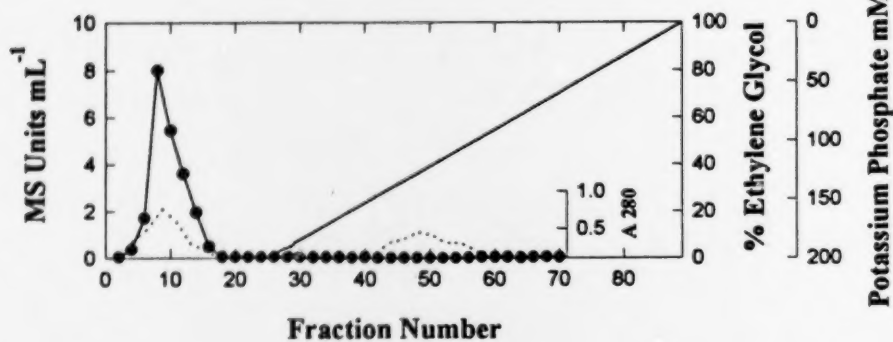
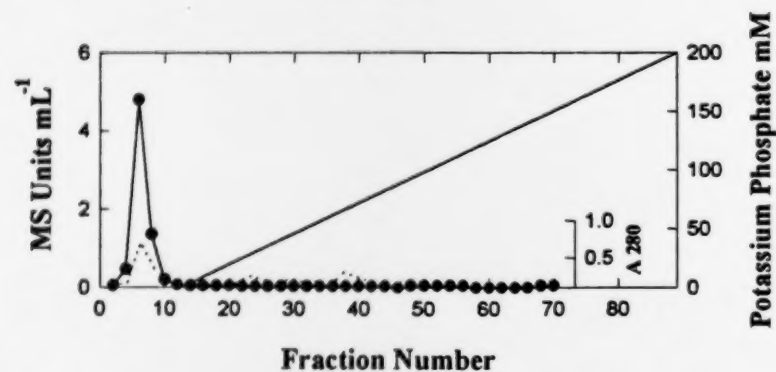


FIGURE 3.21. Progress of the MS purification from megagametophytes 10 DAI. Pooled fractions from each of the purification steps were subjected to 12% SDS-PAGE and visualized by staining proteins with either Coomassie blue R (lanes A-F) or silver (lane G). The amount of protein loaded in each lane is indicated in parentheses. Lane A, acetone extraction (crude homogenate) (10 μ g); lane B, 0-35% (saturation) $(\text{NH}_4)_2\text{SO}_4$ fraction (15 μ g); lane C, MS peak activity fractions from gel filtration on Sepharose CL-6B (8 μ g); lane D, DE-52 active fractions (8 μ g); lane E, phenyl-Sepharose CL-4B active fractions (7 μ g); lane F, pooled purified MS from flow through fractions on HTP (1.5 μ g); and lane G, silver-stained pooled purified MS fractions from HTP (1.5 μ g). Numerical values to the right of the gel are the molecular masses (in kDa) of protein standards.

FIGURE 3.22. Purification of MS. Elution profiles of MS enzyme activity (•) and absorbance at 280 nm (.....) during (A) Sepharose CL-6B gel filtration, (B) DE-52 cellulose anion-exchange, (C) phenyl-Sepharose CL-4B hydrophobic interaction chromatography, and (D) HTP. For all columns only peak MS activity fractions were collected, pooled, and used for subsequent enzyme purification steps.

A**B****C****D**

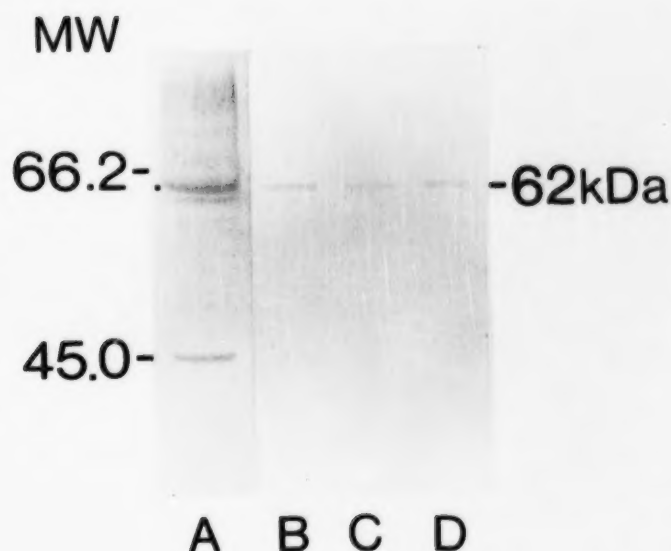
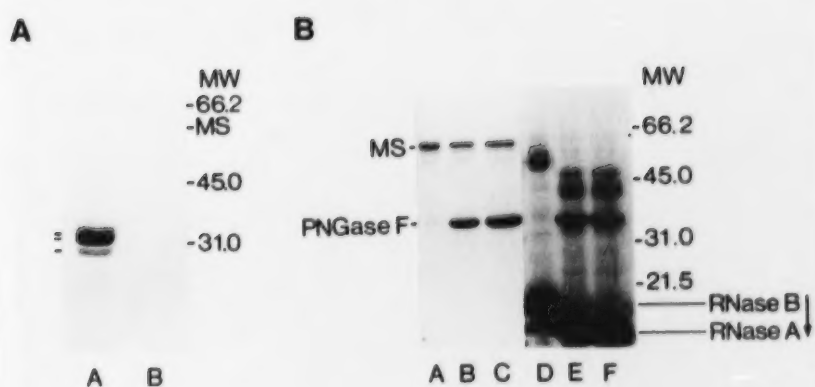


FIGURE 3.23. Immunoblot of loblolly pine proteins probed with polyclonal antiserum to MS and immuno-affinity purified MS IgGs. Protein extracts were separated by SDS-PAGE, electroblotted overnight to nitrocellulose, and probed with either loblolly pine MS anti-serum (lane A) or immuno-affinity purified MS IgGs (lanes B-D). As shown in lane A, antisera prepared from purified MS protein reacted with the 62 kDa MS species as well as a number of other polypeptides. MS IgGs recognizing loblolly pine MS were immuno-affinity purified by the method of Smith and Fisher (1984). Following preparatory SDS-PAGE and standard immunoblotting, the band corresponding to purified pine MS (62 kDa) was excised and IgGs eluted as described in 2.12. MS-specific IgGs were then used to probe protein blots of extracts from purified MS (lane B), glyoxysomes isolated from 10 DAI megagametophytes (lane C), and seed megagametophytes (10 DAI) (lane D). Numerical values to the left of the blot are the molecular masses (in kDa) of protein standards.

FIGURE 3.24. Glycosylation analysis of MS. **(A).** Mature castor bean endosperm cell-free extracts (lane A) and partially purified loblolly pine MS after phenyl-Sepharose CL-4B chromatography (lane B) were subjected to 12% SDS-PAGE and stained for glycoproteins with Schiff's reagent (Fairbanks et al., 1971). Shown in lane A is the glycoprotein staining of lectin RCA₁ (34 and 30 kDa) and ricin_D (33 and 30 kDa) from mature castor bean seeds (Lord, 1985), used here as a Schiff's staining control. For lane A and lane B, 20 µg and 2.5 µg of protein was loaded, respectively. Indicated to the right of the gel is the relative migration of purified MS protein as determined by staining a portion of the gel with Coomassie blue R. Numerical values to the right of the gel are the molecular masses (in kDa) of protein standards. **(B).** SDS-PAGE analysis of loblolly pine MS treated with PNGase F. 2.5 µg of partially purified MS after phenyl-Sepharose CL-4B chromatography (lanes A-C) and 10 µg RNase B (lanes D-F) were incubated with PNGase F (2000 units) for 1 h (lanes B and E), 20 h (lanes C and F), or with no enzyme (lanes A and D) at 37°C. Following incubation, samples were boiled in Laemmli buffer for 5 min and subjected to 12% SDS-PAGE and Coomassie blue R staining. Indicated to the left of the gel is the molecular mass of MS (62 kDa) and PNGase F (36 kDa). Incubation with PNGase F resulted in no mobility shift in the molecular mass of MS on SDS-PAGE (lanes B and C). Indicated to the right of the gel is the relative molecular mass of the RNase B/RNase A control PNGase F deglycosylation reaction. Incubation with PNGase F for 1 h (lane E), or 20 h (lane F) at 37°C, resulted in the deglycosylation of RNase B to RNase A as shown by the mobility shift of the protein's molecular mass from 14.3 kDa (RNase B) to 13.7 kDa (RNase A) on SDS-PAGE. Numerical values to the right of the gel **(B)** are the molecular mass (in kDa) of protein standards.



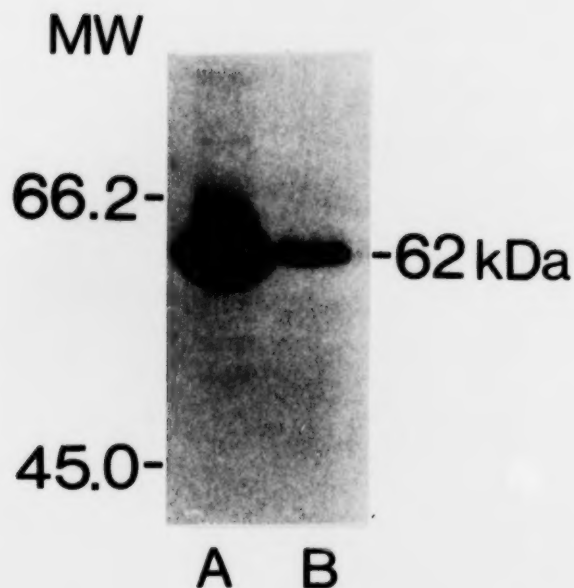


FIGURE 3.25. SDS-PAGE and fluorography of *in vivo* and *in vitro* synthesized immunoselected MS. Protein extracts from ten 8 DAI megagametophytes labelled for 3 h with [^{35} S]-methionine (lane A), or [^{35} S]-methionine labelled *in vitro* translations of 8 DAI poly(A) $^{+}$ RNA (lane B), were immunoselected with cotton MS antiserum and subjected to 12% SDS-PAGE and fluorography. Indicated to the right of the fluorograph is the relative migration of purified MS protein as determined by staining a portion of the gel with Coomassie blue R. Numerical values to the left of the fluorograph are the molecular masses (in kDa) of protein standards.

reacting contaminating polypeptides was a dominant protein with a molecular mass of 45 kDa. To obtain IgGs that were monospecific for loblolly pine MS an antibody immunoaffinity purification procedure (Smith and Fisher, 1984) was carried out. Figure 3.23 illustrates that a single 62 kDa polypeptide was observed on immunoblots of purified MS protein (lane B), 10 DAI megagametophyte glyoxysomal fraction (lane C), and post-germinated megagametophytes (10 DAI) (lane D), indicating that MS did not undergo any change in its monomeric molecular mass during purification.

Loblolly pine MS does not appear to be a glycoprotein. The purified enzyme did not bind immobilized Con A (data not shown), react with Schiff's reagent (Figure 3.24 A), nor was it specifically deglycosylated by PNGase F (Figure 3.24 B). Moreover, MS immunoprecipitated from the products of 8 DAI *in vivo* and *in vitro* [^{35} S]-methionine labelled translations yielded similar polypeptides with molecular masses of that of the purified enzyme (62 kDa) (Figure 3.25).

3.4. Construction of Loblolly Pine Cotyledon and Megagametophyte cDNA Libraries

In order to begin to investigate the developmental regulation of enzymes involved in reserve mobilization in loblolly pine seeds, λ ZAP cDNA libraries were constructed from poly(A)⁺ RNA isolated from germinated megagametophytes and embryos (Table 3.4). In this thesis, only experiments dealing with the isolation of glyoxysomal enzyme clones from the megagametophyte cDNA library are presented (see 3.5 and 3.6). However in this section (3.4), the construction of a loblolly pine cotyledon cDNA library is discussed, since in future studies it will be used to isolate, and subsequently examine embryo-localized enzymes such as glycolate oxidase and arginase following germination.

For megagametophyte and cotyledon λ ZAP cDNA library construction, poly(A)⁺ RNA was purified from total RNA extracted from 8 DAI isolated megagametophytes and 9-10 DAI isolated cotyledons. Because of the numerous reports describing the recalcitrant nature of total RNA and poly(A)⁺ RNA preparations from conifers (Baker et

TABLE 3.4. Summary of 8 DAI megagametophyte and 9-10 DAI cotyledon λ ZAP cDNA library construction

Poly(A) ⁺ RNA source (5 μ g)	8 DAI megagametophytes	9-10 DAI cotyledons
First strand cDNA synthesis incorporation rates ^a (% conversion of mRNA to cDNA)	24.8%	35.9%
Second strand cDNA synthesis incorporation rates ^a (% conversion of cDNA to double-stranded cDNA)	75.1%	83.9%
Total # of primary recombinant clones ^b	1.4×10^6	2.5×10^6
# of primary recombinant clones per μ g of poly(A) ⁺ RNA ^c	2.8×10^5	5.0×10^5
Total # of recombinant clones following library amplification ^d	2.0×10^{10}	3.2×10^{10}
Percentage of recombinants ^e	> 99%	> 99%
Average cDNA insert size ^f	1.2 kB	1.45 kB

- a First and second strand cDNA synthesis was carried out in the presence of $\alpha[^{32}\text{P}]$ -dCTP. Aliquots from each reaction were measured for the amounts of $\alpha[^{32}\text{P}]$ -dCTP incorporated into DNA by TCA precipitation. Based on 5 μg of poly(A)⁺ RNA as a template, values shown are the percent conversion of mRNA to cDNA during first strand cDNA synthesis, and the percent conversion cDNA to double-stranded cDNA during second strand cDNA synthesis. Percentages are the mean of duplicate TCA precipitations.
- b Following *in vitro* packaging, the total number of primary recombinant clones in the megagametophyte and cotyledon cDNA libraries was determined by titering and plating an aliquot of the library on NZY agar plates with *E. coli* XL1-Blue MRF'. The total number of primary recombinant clones was determined by dividing the number of plaques observed on plates, the dilution factor, and the entire packaging volume, by the amount of cDNA/UNI-ZAP XR vector DNA packaged and the volume plated.
- c Total amount of primary recombinant clones (see b) divided by the total amount of poly(A)⁺ RNA (5 μg) used as a template for first strand cDNA synthesis.
- d To establish a permanent or stable supply of the library, 10⁶ recombinants from both megagametophyte and embryo primary cDNA libraries were amplified on NZY agar plates. The total number of recombinant clones following library amplification was determined using the same calculation for determining the number of primary recombinant clones (see b).
- e The percentage or ratio of recombinant versus nonrecombinant background plaques was determined by plating an aliquot of the megagametophyte or embryo cDNA libraries on NZY agar and carrying out blue-white color selection using IPTG-X-Gal. The amino terminus sequences of the *lacZ* gene within the UNI-ZAP XR cloning vector used for the construction of both libraries provides α -

complementation with the *E.coli* XL-1 Blue MRF' host strain. Insertion of a cDNA clone into the polylinker region of the UNI-ZAP XR vector disrupts the *lacZ* gene, resulting in no expression of functional β -galactosidase, and therefore white plaques. Nonrecombinant background plaques are blue as result of α -complementation and the production of a enzymatically active β -galactosidase protein.

- ' To determine the average cDNA insert size for both the megagametophyte and cotyledon cDNA libraries, 10 plaques were randomly picked from NZY agar plates from each library and λ DNA isolated. The 10 λ DNA samples from each library were then digested with EcoR I and Kpn I, and the excised cDNA inserts analyzed by 0.8% agarose gel electrophoresis and ethidium bromide staining. The size of cDNA inserts was determined by comparing their relative mobility to DNA standards of known molecular weights.

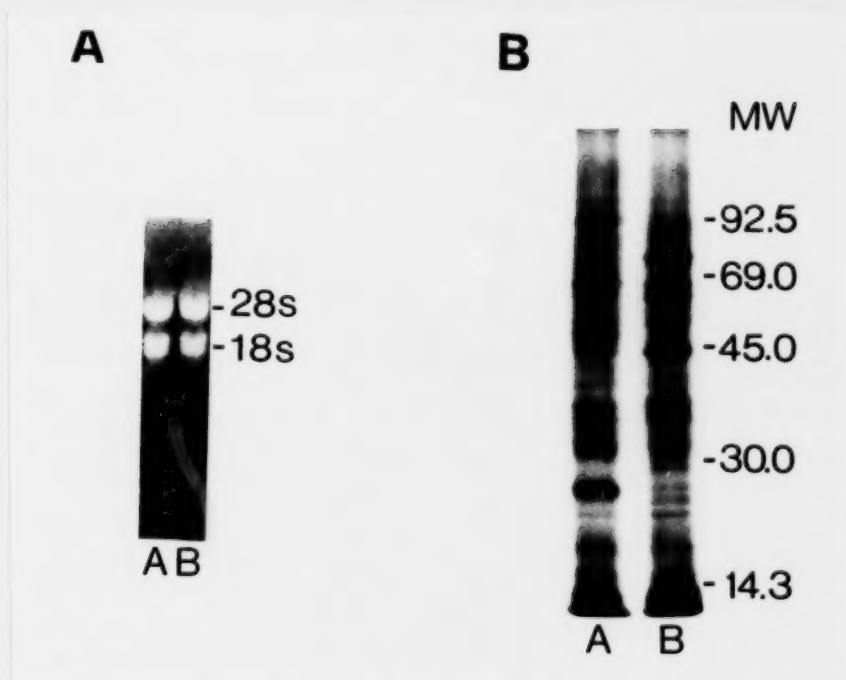


FIGURE 3.26. Analysis of megagametophyte and cotyledon RNA preparations for subsequent cDNA synthesis. (A). Total RNA isolated from 8 DAI megagametophytes (lane A) and 9-10 DAI cotyledons (lane B) was subjected to 1.2% (v/v) formaldehyde gel electrophoresis and stained with ethidium bromide. For both lanes 10 μ g of total RNA was loaded. Indicated to the right of the figure is the relative migration of 28S and 18S rRNA standards. (B). SDS-PAGE/fluorograph of *in vitro* translation products from poly(A)⁺ RNA purified from 8 DAI megagametophytes (lane A) and 9-10 DAI cotyledons (lane B). Poly(A)⁺ RNA was purified from total RNA from isolated megagametophytes and embryos and translated in a rabbit reticulocyte cell-free translation system with [³⁵S]-methionine. *In vitro* synthesized proteins were then analyzed by SDS-PAGE/fluorography. For each gel 10,000 dpm was loaded. Indicated to the right of the gel is the molecular weights (in kDa) of [¹⁴C]-methylated protein standards.

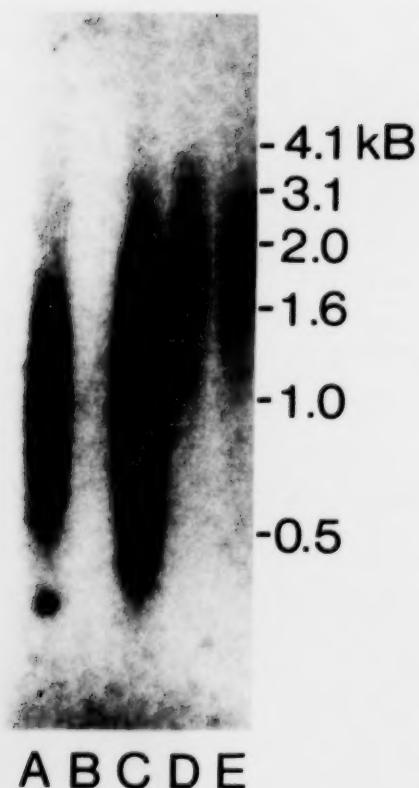


FIGURE 3.27. Alkaline agarose gel electrophoresis of first and second strand cDNAs synthesized from poly(A)⁺ RNA isolated from 8 DAI megagametophytes and 9-10 DAI cotyledons. α [³²P]-dCTP labelled megagametophyte and cotyledons first (lane A and C) and second strand (lanes D and E) cDNA products were subjected to 1.4% alkaline agarose gel electrophoresis. Lane B is empty. Following electrophoresis, the nucleic acids were fixed in the gel with 7% (v/v) TCA for 30 min, dried on Whatman 3MM paper overnight, wrapped in saran wrap, and then exposed to X-ray film at -70°C with intensifying screens overnight. For all lanes 50,000 dpm of incorporated radioactivity was loaded. The decrease in the relative intensity between the first (lanes A and C) and second (lanes D and E) cDNA strands is due to the relative ratio of α [³²P]-dATP to the amount of NTP in the first and second strand reaction. Indicated to the right of the figure is the relative migration of DNA standards (in kB), obtained by cutting a portion of the gel and staining with ethidium bromide.

al., 1992; Bahloul and Burkard, 1993; Graham, 1993; Martin et al., 1993; Schultz et al., 1994), it was necessary to demonstrate the purity and integrity of our RNA samples before carrying out time-intensive cDNA synthesis. Typically, the RNA extraction's yielded an absorbance of $A_{230}/A_{260}/A_{280}$ of 1:2:1 indicating that most of the contaminating polysaccharides and proteins in the megagametophyte and embryo were removed (data not shown). Electrophoresis of megagametophyte and cotyledon total RNA on 1.2% (v/v) formaldehyde gels stained with ethidium bromide showed that the bands corresponding to the 28S and 18S rRNA species were clearly resolved, with little or no low molecular weight degraded RNA's detectable (Figure 3.26 A). The high quality of our cotyledon and megagametophyte RNA preparations used for cDNA synthesis was further demonstrated by the ability of the poly(A)⁺ RNA to synthesis a wide range (14-90 kDa) of [³⁵S]-methionine labelled polypeptides in a rabbit reticulocyte *in vitro* translation system (Figure 3.26 B). SDS-PAGE and fluorography of the immunoprecipitation products of CAT and ICL antiserum incubated with a megagametophyte [³⁵S]-labelled *in vitro* translation mixture revealed that both polypeptides were present at relatively high levels (data not shown). This observation indicated that the mRNA species encoding these proteins are present during this stage of megagametophyte development (i.e. post-germination), and that these RNA samples would therefore direct the synthesis of a cDNA library enriched with CAT and ICL messages.

Ultimately, the final test of the purity and integrity of the megagametophyte and cotyledon RNA samples for library construction was judged during cDNA synthesis. Measurements of the incorporation rates during first and second strand cDNA synthesis by TCA precipitation indicated that 24.8% and 35.9% of the mRNA was converted to cDNA, and 75.1% and 83.9% of the cDNA was converted to double-stranded cDNA in the megagametophyte and cotyledon, respectively (Table 3.4). The efficiency of cDNA synthesis was also analyzed by alkaline gel electrophoresis. Shown in Figure 3.27 is an autoradiograph of megagametophyte and cotyledon first and second strand synthesized cDNAs subjected to alkaline agarose gel electrophoresis. Both megagametophyte (lane A) and cotyledon (lane C) poly(A)⁺ RNA preparations were able to direct the synthesis of first strand cDNA transcripts ranging from 350 to 4000 bases. Similarly, the distribution

of megagametophyte (lane D) and cotyledon (lane E) second strand cDNAs ranged from 600-4000 bases (Figure 3.27). This similarity in the size distribution of first and second strand megagametophyte and cotyledon cDNAs indicated that no detectable self-priming or 3' loop formation of the first strand molecule occurred during second strand cDNA synthesis.

Following cDNA synthesis and EcoR I adaptor ligation, the cDNA molecules were directionally cloned into the UNI-ZAP XR vector in the sense orientation, and assembled into phage particles *in vitro*. The resultant recombinant phage were then used to infect the *E.coli* host XL1-Blue MRF' and plated for titer. A total of 1.4×10^6 and 2.5×10^6 primary recombinant clones were obtained for the megagametophyte and cotyledon libraries, respectively. Following amplification, the total number of recombinants in the megagametophyte and cotyledon libraries were 2.0×10^{10} and 3.2×10^{10} , respectively (Table 3.4). Blue-white screening with IPTG-X-Gal indicated that over 99% of the recombinants in both libraries contained cDNA inserts (Table 3.4). Furthermore, a random selection of 10 plaques from each library, followed by DNA isolation and restriction endonuclease digest analysis, indicated that the average cDNA insert size was 1.2 kB and 1.45 kB for the megagametophyte and cotyledon library, respectively (Table 3.4).

3.5. Isolation and Characterization of Two cDNAs Encoding ICL

Approximately 2.5×10^5 independent clones of the 8 DAI megagametophyte λ ZAP cDNA library were transferred to nitrocellulose filters and screened with ICL antiserum. After four rounds of screening, eight purified positive clones were *in vivo* excised from λ into pBluescript and analyzed by EcoR I/Kpn I digestion and agarose gel electrophoresis (Figure 3.28). Overall, five of the clones contained cDNA inserts over 2 kB and the other three contained inserts ranging from 1.3-1.5 kB. Similarities and

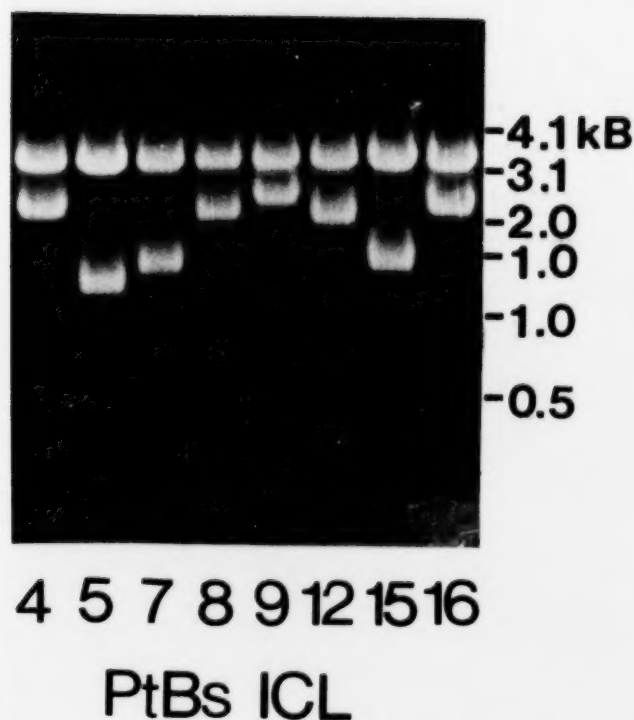


FIGURE 3.28. Agarose gel electrophoresis of ICL cDNA clones. A total of eight positive clones (designated PtBs ICL 4, 5, 7, 8, 9, 12, 15, and 16) were obtained from the immunoscreening of a 8 DAI megagametophyte λ ZAP cDNA library with ICL antiserum. Following *in vivo* excision, each cDNA clone was subcloned into pBluescript, digested with EcoR I and Kpn I, and analyzed by 0.8% agarose gel electrophoresis and ethidium bromide staining. Indicated to the right of the figure is the relative migration of DNA standards (in kB). Except the first 1.0 kB marker should be 1.5 kB.

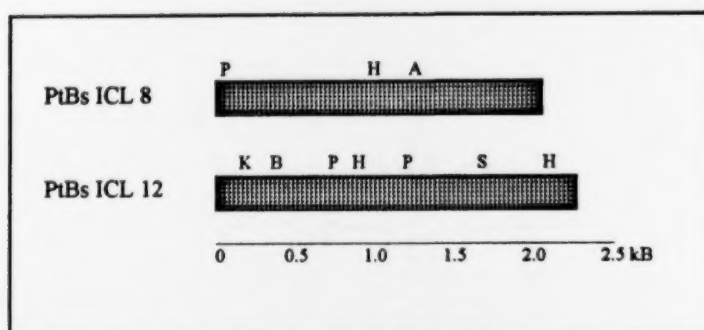


FIGURE 3.29. Restriction endonuclease maps of PtBs ICL 8 and PtBs ICL 12. PtBs ICL 8 and PtBs ICL 12, representing members of two distinct groups of ICL cDNA clones obtained from immunoscreening of a megagametophyte λ ZAP cDNA library with ICL antiserum, were mapped with the following restriction endonucleases: A, Apa I; B, Bst I; H, Hind III; K, Kpn I; P, Pst I; S, Sac I.

differences in the restriction endonuclease patterns indicated that the cDNA clones, designated as PtBs ICL 4, 5, 7, 8, 9, 12, 15 and 16, were members of two distinct groups (data not shown). Furthermore, partial sequencing of 5' and 3' ends of the five longest cDNA clones indicated that PtBs ICL 4, 8, and 16 were the same clone, and PtBs ICL 9 was a slightly truncated version of PtBs ICL 12 (data not shown). Of these, the longest cDNA clones from each group, PtBs ICL 8 (2 kB) and PtBs ICL 12 (2.1 kB), were chosen and sequenced to entirety. Figure 3.29 is a comparison of the restriction endonuclease maps of PtBs ICL 8 and PtBs ICL 12, and Figures 3.30 and 3.31 are the complete nucleotide and deduced amino acid sequences for both ICL cDNAs. PtBs ICL 8 is 2017 bp in length with a 75 bp 5' untranslated region, a 202 bp 3' untranslated region, and a 1740 bp open reading frame, or a deduced amino acid sequence of 580 residues (protein molecular weight 64 684 Da). The 3' untranslated region contains two consensus polyadenylation signals, AATAA (nucleotides 1943-1948 and 1959-1963) (Joshi, 1987) and a 17 bp poly(A)⁺ tail (nucleotides 2001-2017) (Figure 3.30). PtBs ICL 12 is 2176 bp in length with a 68 bp 5' untranslated region, a 389 bp 3' untranslated region, and a 1719 bp open reading frame, or a deduced amino acid sequence of 573 residues (protein molecular weight 64 279 Da). The 3' untranslated region contains a consensus polyadenylation signal, AATAA (nucleotides 2137-2141) (Joshi, 1987) and a 19 bp poly(A)⁺ tail (nucleotides 2157-2176) (Figure 3.31). The sequences CATCATGGC (nucleotides 72-80) and CTTCATGGC (nucleotides 65-73)-surrounding the first initiating methionine ATG in PtBs ICL 8 and PtBs ICL 12, respectively, show a high degree of identity with the consensus sequence surrounding the ATG initiation codon for plants (Kozak, 1984; Lutcke et al., 1987).

Shown in Figure 3.32 is a comparison of the deduced amino acid sequences of PtBs ICL 8 and PtBs ICL 12 to that of castor bean (Beeching and Northcote, 1987), *Brassica napus* (Comai et al., 1989), cotton (Turley et al., 1990), cucumber (Reynolds and Smith, 1995) and *E.coli* (Matsuoda and McFadden, 1988). PtBs ICL 8 and PtBs ICL 12 show a 79% similarity in their deduced amino acid sequences, as well as a 73% nucleotide similarity within their coding regions. Overall, the deduced amino acid sequences for both loblolly pine ICLs are highly similar to the sequences reported for

FIGURE 3.30. Nucleotide and deduced amino acid sequences of PtBs ICL 8. The cDNA clone encoding loblolly pine ICL was isolated from a λ ZAP cDNA library constructed from poly(A)⁺ RNA extracted from 8 DAI megagametophytes. The complete sequence was obtained by sequencing both strands of PtBs ICL 8 (coding and non-coding) using synthetic oligonucleotide primers designed from sequence obtained in the previous round of sequencing (Table 2.1). Nucleotides are numbered in the left-hand column and amino acids in the right-hand column. The termination codon (TAA) is marked (*), and the putative polyadenylation signals are underlined.

FIGURE 3.31. Nucleotide and deduced amino acid sequences of PtBs ICL 12. The cDNA clone encoding loblolly pine ICL was isolated from a λ ZAP cDNA library constructed from poly(A)⁺ RNA extracted from 8 DAI megagametophytes. The complete sequence was obtained by sequencing both strands of PtBs ICL 12 (coding and non-coding) using synthetic oligonucleotide primers designed from sequence obtained in the previous round of sequencing (Table 2.1). Nucleotides are numbered in the left-hand column and amino acids in the right-hand column. The termination codon (TAA) is marked (*), and the putative polyadenylation signal is underlined.

FIGURE 3.32. Comparison of the deduced amino acid sequences of ICL from loblolly pine and other ICLs. The predicted amino acid sequence of pine (*Pinus taeda*) (PtBs ICL 8 and PtBs ICL 12) was compared to castor bean (*Ricinus communis*) (Beeching and Northcote, 1987), *Brassica napus* (Comai et al., 1989), cotton (*Gossypium hirsutum*) (Turley et al., 1990), cucumber (*Cucumis sativus*) (Reynolds and Smith, 1995), and *E. coli* (Matsuoda and McFadden, 1988). Sequences were obtained from Genbank and comparison of deduced amino acid sequences was carried out using the PC/Gene Clustal similarity search program. Four conserved domains are underlined. Gaps (dashes) in sequences are introduced to maximize alignment. Asterisks below each line show where all plant ICL amino acid residues are identical, and the amino acid residues of the active center are indicated above the line (•).

Loblolly Pine 8	MAIYSAQEN	SILSEAEFE	AEVSETOAM	NSDLPFLTR	RYTARDVR	LRGSHQSTA	SNEMAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
Loblolly Pine 12	MAIYVQ-GAS	MMIDEAKIE	AEVAEAMAM	NS-ERFLUTQ	RYTAKDVR	LRGTHQSTA	SNELAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
Castor Bean	MAASF-GPS	MIMEEGREE	AEVAEVOAM	NS-ERFLUTQ	RYTAKDVR	LRGTHQSTA	SNELAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
Brassica napus	MAASF-VPS	MIMEEGREE	AEVAEVOAM	SS-ERFLUTQ	RYTAKDVR	LRGTHQSTA	SNEMAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
Cotton	MAASF-VPS	MIMEEGREE	TEVAEVOAM	NS-ERFLUTQ	RYTAKDVR	LRGTHQSTA	SNEMAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
Cucumber	MAASF-VPS	MIMEEGREE	AEVAEVOAM	NS-ERFLUTQ	RYTAKDVR	LRGTHQSTA	SNEMAKLWR	TLKTHQAKT	ASRTFGALDP	VVUSHAKYL
E. coli	MASTRQ--I	ELQKEM--	---	---	---	---	---	---	---	---
Loblolly Pine 8	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
Loblolly Pine 12	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
Castor Bean	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
Brassica napus	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
Cotton	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
Cucumber	DTIYVGMQC	SSTHTTNEP	GPDLADYFD	TVPNKVEHLF	FAOQHGRKQ	KEARMSHRE	ERSTPYDY	LKPIIADGDT	FGGATATYK	LCKLFVERGA
E. coli	EAVTSGNQV	ADANLAASH	YEDQSLPAN	SVPAVVERIN	NTRADQIQ	MSAGI	---	---	---	---
Loblolly Pine 8	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
Loblolly Pine 12	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
Castor Bean	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
Brassica napus	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
Cotton	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
Cucumber	AGVHIEDQAS	VTKKCGHAG	KVLVSVEHV	NRMVAARQF	DIMGVETLV	ARTDAVAATL	IQTNVDARQ	QFILGATNEN	LGRPLAUVL	ARMAHSGKSG
E. coli	AAVHEDQLA	SVKCGHGG	KVLVPTQAI	QCLVAARLA	DVTGVETLV	ARTDAVAATL	ITSDCDPYDS	EFITGE	---	---
Loblolly Pine 8	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Loblolly Pine 12	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Castor Bean	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Brassica napus	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Cotton	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Cucumber	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
E. coli	ADLQAVEDEN	HAMADLGLFS	DCVVDGKOL	NYSDEKERR	LGMHARTG	NTGNVLSYTO	AKELAEKGI	SNLFDMDOLP	RTREGFYRQ	GSVMAIVRG
Loblolly Pine 8	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Loblolly Pine 12	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Castor Bean	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Brassica napus	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Cotton	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Cucumber	MAFGPHADII	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
E. coli	LAYAPADLV	WETSSPDV	ECRDFALGVK	SKHPEMLAY	NLSFPMQDA	SMHTEQMK	FIPEIARLGY	CHQITLAGE	HADALVDTF	AKDFAQRGHL
Loblolly Pine 8	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
Loblolly Pine 12	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
Castor Bean	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
Brassica napus	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
Cotton	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
Cucumber	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---
E. coli	ATYKIQORE	MMGVDTLH	QMSGANYTD	QILLATVGGI	SATANAKGV	TEQFEETOS	STLALESNTG	AGTVLAKSH	---	---

other higher plants (Figure 3.32). PtBs ICL 8 exhibits identities of 77, 76, 79, 79 and 38% to castor bean, *Brassica napus*, cotton, cucumber and *E.coli* ICL, respectively. PtBs ICL 12 exhibits identities of 75, 75, 76, 77 and 38% to castor bean, *Brassica napus*, cotton, cucumber and *E. coli* ICL, respectively. Alignment of the prokaryotic enzyme's amino acid sequence with those from higher plants, including loblolly pine, revealed a large gap starting at amino acid 254 and extending over 103 amino acids (Figure 3.32). Further analysis of the aligned ICL sequences showed four domains of high identity underlined in Figure 3.32. For the *E.coli* enzyme, Lys¹⁹³ and Cys¹⁹⁵, both located within the first of these regions, as well as Ser³¹⁹ and Ser³²¹, situated in the third conserved domain, were identified as essential for catalysis (Nimmo et al., 1989; Ko et al., 1992; Diehl and McFadden, 1993). In accordance with these findings, both PtBs ICL 8 and PtBs ICL 12 deduced amino acid sequences possess identical amino acids at these respective sites, further indicating the importance of these amino acids in catalysis.

3.6. Isolation and Characterization of a cDNA Encoding the 5' half of CAT

λ DNA isolated from the 8 DAI megagametophyte cDNA library (section 3.4) was used as a template for PCR amplification of a cDNA clone encoding the protein coding region of CAT. Four degenerate primers, designated pCAT 1, pCAT 2, pCAT 3 and pCAT 4 (Table 3.5) were synthesized based on highly conserved domains of known plant CAT sequences including maize (Redinbaugh et al., 1989), cotton (Ni et al., 1990; Ni and Trelease, 1991), pea (Isin and Allen, 1991), tomato (Drory and Woodson, 1992), and *Arabidopsis* (Chevalier et al., 1992). PCR was performed in two separate reactions of (1) pCAT 1 (sense primer) in combination with pCAT 3 (antisense primer) for the amplification of a segment of CAT cDNA corresponding to the 5' half of the protein coding region, and (2) pCAT 2 (antisense primer) in combination with pCAT 4 (sense primer) for the amplification of a segment of CAT cDNA corresponding to the 3' half of the protein coding region (Figure 3.33). The PCR for the 5' half of the CAT gene

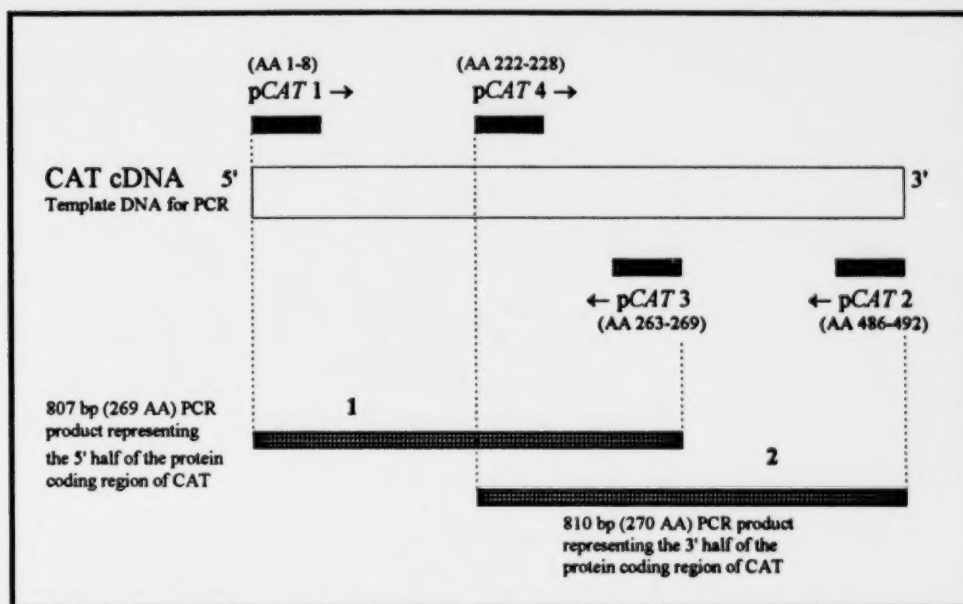


FIGURE 3.33. Schematic of the cloning strategy for loblolly pine CAT with the PCR. λ DNA was isolated from a 8 DAI megagametophyte cDNA library. This DNA was used in a two separate PCR reactions with (1) degenerate CAT primers pCAT 1 (sense, amino acids 1-8; MDPYKHRP) and pCAT 3 (antisense, amino acids 263-269; NYPEWKL), designed based on highly conserved domains of plant CAT to amplify the 5' half of the protein coding region of CAT (807 bp, 269 amino acids), or with (2) degenerate CAT primers pCAT 4 (sense, amino acids 222-228; VKFHWKP) and pCAT 2 (antisense, amino acids; LNVRPSI) designed based on highly conserved domains of plant CAT to amplify the 3' half of the protein coding region of CAT (810 bp, 270 amino acids).

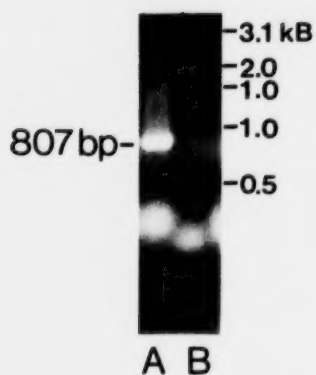


FIGURE 3.34. Agarose gel electrophoresis of CAT PCR products. λ DNA isolated from a 8 DAI megagametophyte cDNA library was a template for the PCR of the 5' (lane A) and 3' (lane B) halves of the CAT gene using pCAT 1 and pCAT 3, and pCAT 2 and pCAT 4 as primers, respectively. Following PCR, amplification products were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining. Numerical values to the right of the figure is the relative migration of DNA standards (in kB).

produced the expected 807 bp product (Figure 3.34, lane A). However, no product (810 bp) was amplified in the 3' CAT PCR reaction (Figure 3.34, lane B). This result and the our consistent inability to amplify the 3' half of the CAT gene, even when PCR conditions were varied for optimization (i.e., $MgCl_2$ concentration, temperature of primer-template annealing), suggests that the 3' half of the CAT protein coding region may not be as equally conserved in loblolly pine as other known plant CATs examined to date.

Following PCR the 807 bp product (primers pCAT 1 and pCAT 3) was gel purified, cloned into pGEM-T vector, and designated PtGEM 5-*Cat0* (Higo et al., 1994). Shown in Figure 3.35 is the nucleotide and deduced amino acid sequences of PtGEM 5-*Cat0* encoding the 5' half of the protein coding region. The predicted amino acid sequence of loblolly pine CAT shares extensive homology with other plant CATs. Figure 3.36 is a comparison of the deduced amino acid sequence of PtGEM 5-*Cat0* with CAT from cotton (subunit 1, Ni et al. 1990; subunit 2, Ni and Trelease, 1991), *Arabidopsis* (Chevalier et al., 1992), pea (Isin and Allen, 1991), tomato (Drory and Woodson, 1992) and maize CAT 1 (Redinbaugh et al., 1988). The 5' half of loblolly pine CAT protein exhibits identities of 90, 89, 87, 88, 78, and 86% to cotton subunit 1, cotton subunit 2, *Arabidopsis*, pea, tomato, and maize CAT 1, respectively. Of the amino acids located in the 5' half of the CAT protein reported to be involved in catalytic activity (His⁶⁵, Ser¹⁰⁴, and Asn¹³⁸) and direct association with the heme group (Val⁶⁴, Arg¹⁰², Thr¹⁰⁵, and Phe¹⁴³), all are conserved in loblolly pine CAT (Murthy et al., 1981; Fita and Rossmann, 1985) (Figure 3.36).

1	atg gat cct tac aag cat cgt ccc tca agc gcg tac aat tct cct ttc tgg aca acc aac gct ggt gcc cca gtg <u>Met Asp Pro Tyr Lys His Arg Pro Ser Ser Ala Tyr Asn Ser Pro Phe Trp Thr Thr Asn Ala Gly Ala Pro Val</u>	25
76	tgg aat aac aat tcc tca ttg acc atc ggc tca aga ggt cct atc ctt ttg gag gat tat cac ctg gtg gag aag <u>Trp Asn Asn Asn Ser Ser Leu Thr Ile Gly Ser Arg Gly Pro Ile Leu Leu Glu Asp Tyr His Leu Val Glu Lys</u>	50
151	att gca cag ttt gat agg gaa cgt gtg ccg gag aga gtt gtt cat gct aga gca gct agt gcg aag ggt ttt ttc <u>Ile Ala Gln Phe Asp Arg Glu Arg Val Pro Glu Arg Val Val His Ala Arg Gly Ala Ser Ala Lys Gly Phe Phe</u>	75
226	gag gtg acc cat gac att tct cat ctt act tcc gcc gat ttc ctt cgg gca gcc gcc gta cag acc ccc ctg atc <u>Glu Val Thr His Asp Ile Ser His Leu Thr Ser Ala Asp Phe Leu Arg Ala Ala Gly Val Gln Thr Pro Leu Ile</u>	100
301	gtg cgt ttc tca act gtt atc cat gag cgt ggg agt cct gaa acc ctc cga gat cct cgc ggg ttc gct gtg aaa <u>Val Arg Phe Ser Thr Val Ile His Glu Arg Gly Ser Pro Glu Thr Leu Arg Asp Pro Arg Gly Phe Ala Val Lys</u>	125
376	ttc tac acc aga gag ggg aac ttc gat ctt gtt gga aat aat ttt cct gta ttc ttt gtt cgc gat ggt atg aag <u>Phe Tyr Thr Arg Glu Gly Asn Phe Asp Leu Val Gly Asn Asn Phe Pro Val Phe Phe Val Arg Asp Gly Met Lys</u>	150
451	ttt cca gac atg gtt cat gca ttg aaa cca aat ccc aaa agt cac att caa gaa gac tgg agg att cta gat ttc <u>Phe Pro Asp Met Val His Ala Leu Lys Pro Asn Pro Lys Ser His Ile Gln Glu Asp Trp Arg Ile Leu Asp Phe</u>	175
526	ttc tct cac cac cca gag agt ttg cat atg ttc acc ttt ctc ttt gat gat ctt gga atc cct tta aac tac agg <u>Phe Ser His His Pro Glu Ser Leu His Met Phe Thr Phe Leu Phe Asp Asp Leu Gly Ile Pro Leu Asn Tyr Arg</u>	200
601	cat atg gac ggg tct ggt gtc cac acc ttt acc ctt gtt aat agg gct ggg agg caa acc tat gtg aag ttt cat <u>His Met Asp Gly Ser Gly Val His Thr Phe Thr Leu Val Asn Lys Ala Gly Arg Gln Thr Tyr Val Lys Phe His</u>	225
676	tgg agg cat act tgt gga gtt aag tgt cta ttg gat gat gaa gca gtt gta gta ggt ggg aca aac cac agt cat <u>Trp Arg His Thr Cys Gly Val Lys Cys Leu Leu Asp Asp Glu Ala Val Val Val Gly Gly Thr Asn His Ser His</u>	250
751	gca acc aag gat ctc tat gat tct ata gca gca ggc aac tac cct gaa tgg aaa ct- <u>Ala Thr Lys Asp Leu Try Asp Ser Ile Ala Ala Gly Asn Tyr Pro Glu Trp Lys Leu</u>	269

FIGURE 3.35. Nucleotide and deduced amino acid sequences of PtGEM 5-*Cat0*. PtGEM 5-*Cat0* was isolated using cDNA from a megagametophyte λ ZAP cDNA library as template for PCR, with primers pCAT 1 and pCAT 3 (underlined) designed on highly conserved domains of other plant CAT. The complete sequence was obtained by sequencing both strands of PtGEM 5-*Cat0* (coding and noncoding) using synthetic oligonucleotide primers designed from sequence obtained in the previous round of sequencing (Table 2.1). Nucleotides are numbered in the left-hand column and amino acids in the right-hand column.

FIGURE 3.36. Comparison of the deduced amino acid sequences of the 5' half of the protein coding region of CAT from loblolly pine and other plant CATs. The predicted amino acid sequence of the 5' half of pine PtGEM 5-*Cat0* was compared to cotton subunit 1 and subunit 2 (Ni et al., 1990; Ni and Trelease, 1991), *Arabidopsis* (Chevalier et al., 1992), pea (Isin and Allen, 1991), tomato (Drory and Woodson, 1992) and maize CAT 1 (Redinbaugh et al., 1988). Sequences were obtained from Genebank and comparison of deduced amino acid sequences was carried out using the PC/Gene Clustal similarity search program. Gaps (dashes) in sequences are introduced to maximize alignment. Asterisks below each line show are where all CAT amino acid residues are identical, and the amino acid residues of the active center are indicated above the line (•). Amino acids of the distal side involved in heme-binding are underlined.

Loblolly Pine	MDPYKHRPSS	AYNSPFWTTN	SGAPVWNNNS	SLTIGSRGPI	LLEDYHLVEK	IAQFDRERP	60
Cotton 1	MDPYKHRPSS	AFNSPFWTTN	SGAPVWNNNS	SLTVGPRGQY	LLEDYHLVEK	LANFDRERIP	
Cotton 2	MDPYKFRPSS	SFDSPPFWTTN	SGAPVWNNNS	SLTVGARGPI	LLEDYHLVEK	LANFDRERIP	
Arabidopsis	MDPYKYRPAS	SYNSPFFTTN	SGAPVWNNNS	SMTVGPRGLI	LLEDYHLVEK	LANFDRERIP	
Pea	MDPYKHRPSS	AFNSPFWTTN	SGAPVWNNNS	SLTVGSRGPI	LLEDYHLVEK	LAQFDRERIP	
Tomato	MDPSKYRPSS	AYDTPFLTTN	AGGPVYNNVS	SLTVGPRGPV	LLEDYHLVEK	LATFDRERIP	
Maize	MDPYKHRPSS	GSNSPFWTTN	SGAPVWNNNS	ALTVGQRGPI	LLEDYHLVEK	LAQFDRERIP	
	*** * ****	* ***	* * * * *	*** **	***** **	** * * * *	

Loblolly Pine	ERVVHARGAS	AKGFFEVTND	ISHLTSADFL	RAAGVQTPLI	VRFSTVIHER	GSPETLRDRP	120
Cotton 1	ERVVHARGAS	AKGFFEVTND	ISHLTCADFL	RAPGVQTVLI	VRFSTVIHER	GSPETLRDRP	
Cotton 2	ERVVHARGAS	AKGFFEVTND	ISQLTCADFL	RAPGVQTVLI	VRFSTVIHER	GSPETLRDRP	
Arabidopsis	ERVVHARGAS	AKGFFEVTND	ISNLTCADEF	RAPGVQTPVI	VRFSTVIHER	GSPETLRDRP	
Pea	ERVVHARGAS	AKGFFEVTND	ISHLTCADFL	RAPGVQTPVI	VRFSTVIHER	GSPETLRDRP	
Tomato	ERVVHARGAS	AKGFFEVTND	ISHLTCADFL	RAPGAQTPVI	CRFSTVVHER	GSPESIRDIR	
Maize	ERVVHARGAS	AKGFFEVTND	VSHLTCADEF	RAPGVQTVLI	VRFSTVVHER	GSPETLRDRP	
	*****	*****	* * * * *	*** ** *	***** **	***** **	

Loblolly Pine	GFAVKFYTRE	GNFDLVGNNE	PVFFVRDGMK	FPDMVHALKP	NPKSHIQEDW	RILDFFSFHP	180
Cotton 1	GFAVKFYTRE	GNFKLVGNNE	PVFFIRDGMK	FPDMVHALKP	NPKSHIQENW	RILDFFSHHP	
Cotton 2	GFAVKFYTRE	GNFKLVGNNE	PVFFIRDGMK	FPDMVHALKP	NPKSHIQENW	RILDFFSHHP	
Arabidopsis	GFAVKPYTRE	GNFDLVGNNE	PVFFIRDGMK	FPDIVHALKP	NPKSHIQENW	RILDFFSHHP	
Pea	GFAVKFYTRE	GNFDLVGNNE	PVFFVHDGMN	FPDMVHALKP	NPKTHIQENW	RILDFFYNFP	
Tomato	GFAVKFYTRE	GNFDLVGNNE	PVFFNRDAKS	FPDTRALKP	NPKSHIQENW	RILDFFSNFL	
Maize	GFAVKFYTRE	GNFKLVGNNE	PVFFIRDGMK	FPDMVHAFKP	NPKTNLQENW	RIVDFFSHHP	
	*****	** *****	**** *	*** ** *	*** ** *	** ** *	

Loblolly Pine	ESLHMTFLF	DDLGIPLNYR	HMDGSGVHTY	TLVNAKGRQT	YVKFHWKPTC	GVKCLLDDEA	240
Cotton 1	ESLHMTFLF	DDLGVQDYR	HMDGSGVNTY	TLINKAGKAH	YVKFHWKPTC	GVKCLLEDEA	
Cotton 2	ESLHMTFLF	DDIGVPQDYR	HMDGSGVHTY	TLINKAGKSH	YVKFHWKPTC	GVKSLLEDEA	
Arabidopsis	ESLHMTFLF	DDIGIPQDYR	HMDGSGVNTY	MLINKAGKAH	YVKFHWKPTC	GVKSLLEDEA	
Pea	ESLHMTFLF	DDVGVPQDYR	HMDGSGVNTY	TLINKAGKSV	YVKFHWKPTC	GVKCLLEEEA	
Tomato	ESLHTFAFFY	DDVCLPTDYR	HMEGFGVHAY	QLINKEGKAH	YVKFHWKPTC	GVKCMSEEEA	
Maize	ESLHMTFLF	DDVGIPLNYR	HMEGFGVNTY	SLINRDGKPH	LVKFHWKPTC	GVKCLLDNEA	
	***** *	** * * *	* * * * *	* * *	***** **	*** *	

Loblolly Pine	VVVGGTNHSH	ATKDLYDSIA	AGNYPGWKL				269
Cotton 1	IKVGGANHSH	ATQDLYDSIA	AGNYPEWKL				
Cotton 2	IRVGGANHSH	ATQDLYDSIA	AGNYPEWKL				
Arabidopsis	IRLGGTNHSH	ATQDLYDSIA	AGNYPEWKL				
Pea	IQVGGSNHSH	ATKDLYDSIA	AGNYPEWKL				
Tomato	IRVGGTNHSH	ATKDLYDSIA	AGNYPEWKL				
Maize	VTVGGTCHSH	ATKDLYDSIA	AGNYPEWKL				
	** ***	** *****	***** **				

3.7. Developmental Gene Expression of Glyoxysomal Enzymes

3.7.1 Changes in mRNA Populations Following Seed Imbibition

In order to examine the changes in overall patterns of gene expression in loblolly pine seeds following imbibition, poly(A)⁺ RNA was purified from total RNA extracted from megagametophytes and embryos. Purified poly(A)⁺ RNAs were translated *in vitro* in a rabbit reticulocyte cell-free system, and [³⁵S]-methionine labelled protein products examined by two-dimensional electrophoresis and fluorography. Representative fluorographs are shown in Figures 3.37 and 3.38. There were a number of noticeable changes in the patterns of protein synthesized *in vitro* in both the megagametophyte and embryo through all the developmental stages examined. Given some of the limitations of the sensitivity of using *in vitro* translation studies and two-dimensional electrophoresis for examining mRNAs species during development, in this report we have only attempted to identify and describe those 'spots' which display easily observable changes in their relative abundance, or appearance and disappearance.

While a number of proteins were constitutively synthesized *in vitro* during all stages examined (arrowed in Figure 3.37 and 3.38) in the both the megagametophyte and embryo, the most conspicuous changes were those subsets of proteins which either increased or decreased in relative abundance during specific seed developmental stages. For example, one set of proteins was synthesized *in vitro* from messages that were stored in the megagametophyte and embryo of the mature seed (0 DOC) (circled in Figure 3.37 and 3.38). These proteins subsequently declined during stratification at 2°C, and were absent in the germinating seed (2 DAI). Another subset of *in vitro* synthesized megagametophyte and embryo proteins were undetectable, or at very low levels, in the

FIGURE 3.37. Representative fluorographs of IEF/SDS-PAGE profiles of proteins synthesized *in vitro* using poly(A)⁺ RNA extracted from megagametophytes isolated from mature through stratification, germination, and post-germination loblolly pine seeds. Poly(A)⁺ RNA was extracted from megagametophytes isolated from mature seeds (0 DOC), seeds through stratification (7-35 DOC), germination (2 DAI), and post-germination (4-8 DAI) and was translated in a rabbit reticulocyte cell-free translation system with [³⁵S]-methionine. *In vitro* synthesized proteins were then analyzed by IEF/SDS-PAGE/fluorography. For each gel 30,000 dpm was loaded. For first dimension IEF the pH is indicated at the top of the figure. Molecular weights in kDa of methylated protein standards run in the second dimension are indicated to the left of all fluorographs. Marked on fluorographs are the positions of *in vitro* translated proteins from megagametophyte poly(A)⁺ RNA species; constitutively synthesized through all stages of development examined (<); decreasing during stratification (O); synthesized only during stratification (▼); constitutively synthesized during stratification and decreasing following germination (↗); and increasing in synthesis following germination (□).

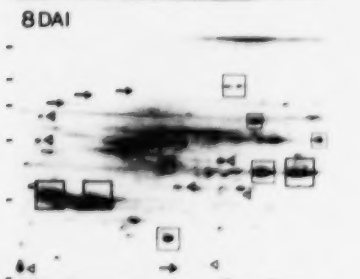
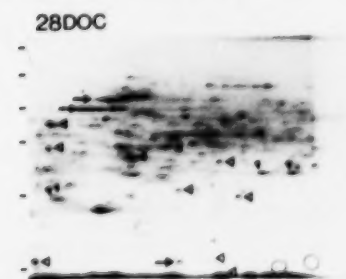
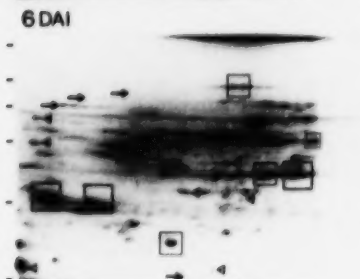
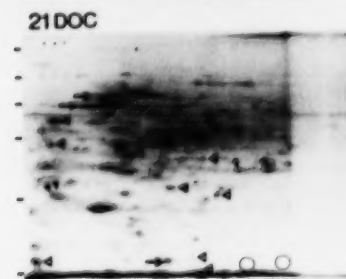
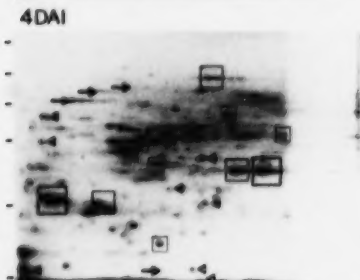
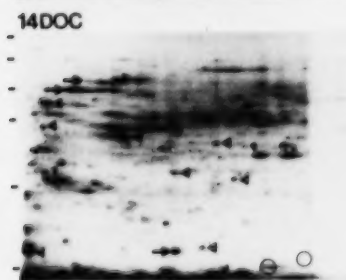
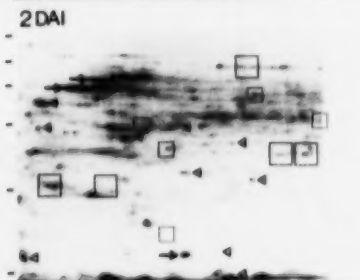
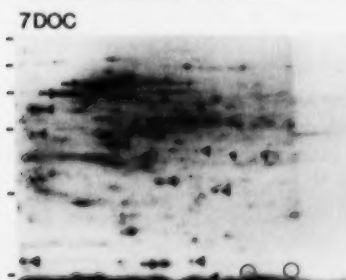
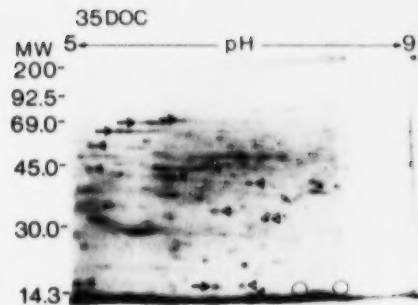
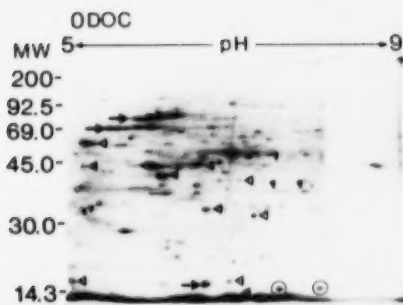
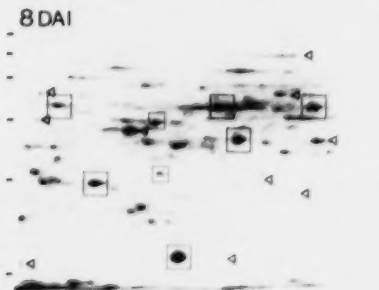
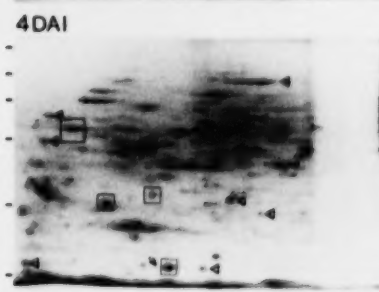
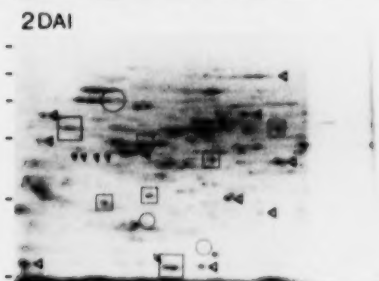
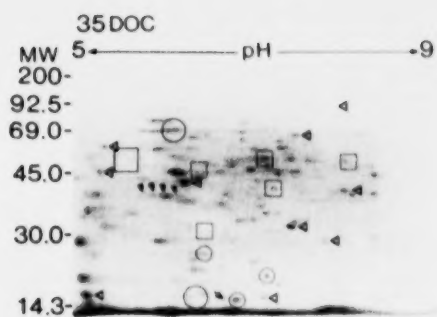
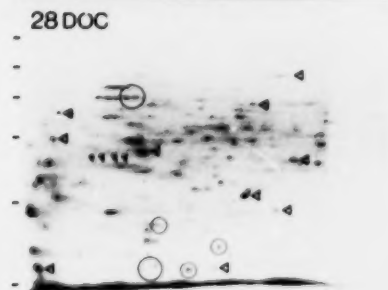
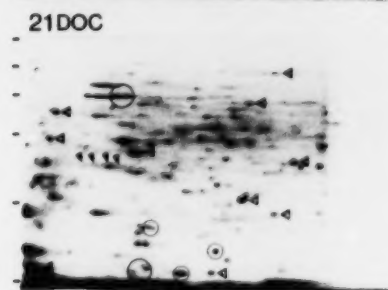
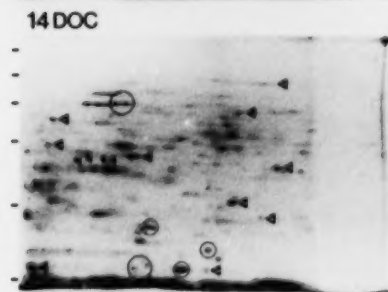
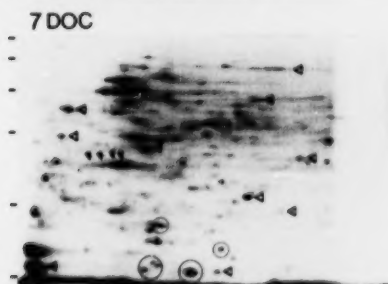
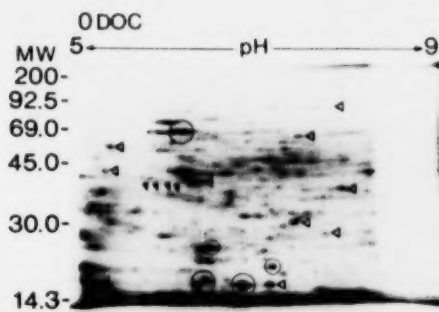


FIGURE 3.38. Representative fluorographs of IEF/SDS-PAGE profiles of proteins synthesized *in vitro* using poly(A)⁺ RNA extracted from embryos isolated from mature through stratification, germination, and post-germination loblolly pine seeds. Poly(A)⁺ RNA was extracted from embryos isolated from mature seeds (0 DOC), seeds through stratification (7-35 DOC), germination (2 DAI), and post-germination (4-8 DAI) and was translated in a rabbit reticulocyte cell-free translation system with [³⁵S]-methionine. *In vitro* synthesized proteins were then analyzed by IEF/SDS-PAGE/fluorography. For each gel 30,000 dpm was loaded. For first dimension IEF the pH is indicated at the top of the figure. Molecular weights in kDa of methylated protein standards run in the second dimension are indicated to the left of all fluorographs. Marked on fluorographs are the positions of *in vitro* translated proteins from embryo poly(A)⁺ RNA species; constitutively synthesized through all stages of development examined (◁); decreasing during stratification (○); synthesized only during stratification (▼); synthesized only during germination (▼); and increasing in synthesis following germination (□).



mature seed. Then during stratification, the relative abundance of these proteins increased and then decreased prior to germination (arrowed in Figure 3.37 and 3.38). The synthesis of this group of proteins peaked at 14 DOC, a time in the stratification period that is correlated with loblolly pine seeds greatest relative increase in germinability (Schneider and Gifford, 1994). In the megagametophyte, one set of *in vitro* synthesized proteins were present in the mature seed, and remained at relatively constant levels during the entire chilling period, but were absent immediately following the completion of seed germination (4 DAI) (Figure 3.37). In the embryo, some proteins, and particularly a low molecular weight species at around 20 kDa, were exclusively synthesized during germination (2 DAI) (Figure 3.38). The messages for these proteins were absent during stratification (0-35 DOC), then increased at seed germination (2 DAI), and disappeared again following radical emergence (4 DAI). Finally, in both the megagametophyte and embryo, a number of proteins which were absent or at very low levels during germination, increased substantially following radicle emergence and into early seedling growth (4-8 DAI) (boxed in Figure 3.37 and 3.38).

3.7.2. MS

MS enzyme activity was assayed from isolated megagametophyte and embryo cell-free extracts taken from mature, stratified, and germinating loblolly pine seeds (Figure 3.39). MS enzyme activity was undetectable in the megagametophyte of the mature seed, rose slightly following stratification ($0.07 \text{ units seed part}^{-1}$) and increased steadily following radical emergence (i.e., completion of germination in loblolly pine seeds, 4 DAI), peaking at 11 DAI ($1.31 \text{ units seed part}^{-1}$) then decreased sharply with tissue senescence. In embryos, MS activity was undetectable in the mature and stratified seed, then rose slightly, peaking at radical emergence ($0.12 \text{ units seed part}^{-1}$) and then declined (Figure 3.39).

To examine the developmental profile of MS steady-state protein levels in the

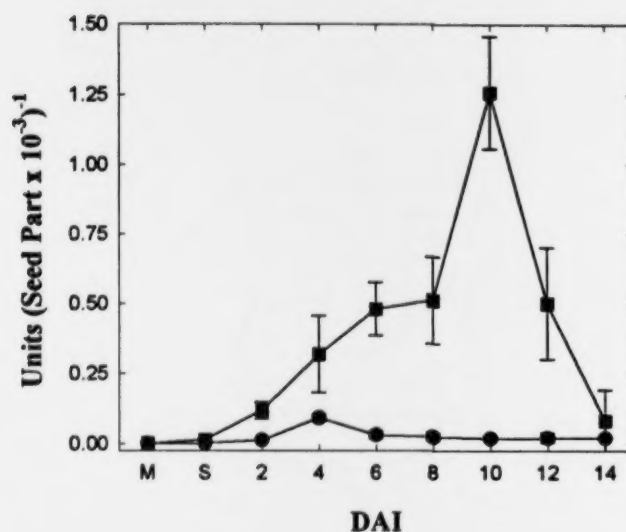
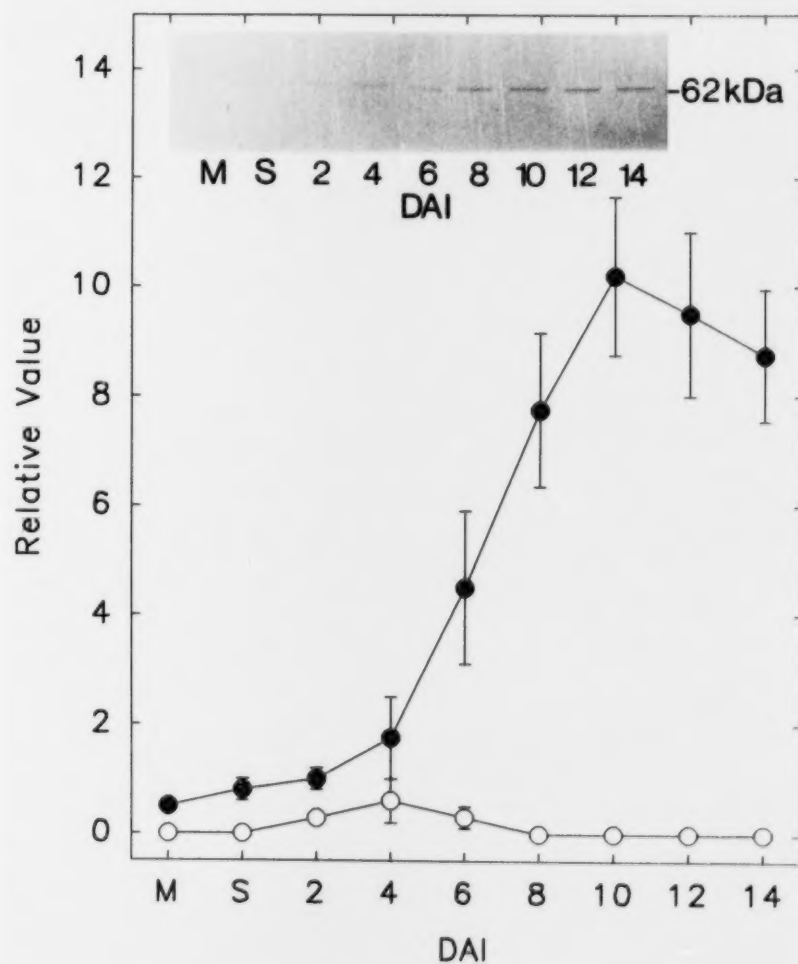


FIGURE 3.39. Profile of MS enzyme activity in megagametophytes and embryos isolated from seeds through stratification, germination, and post-germinative growth. Cell-free extracts from mature (M), stratified (S), and following imbibition at 30°C (2-14 DAI) isolated megagametophytes (■) and embryos (●) were assayed for MS enzyme activity. Each data point is the mean of three determinations $\pm$ SD.

FIGURE 3.40. Relative levels of MS steady-state protein in megagametophytes and embryos isolated from mature, stratified, and post-imbibition seeds. The relative levels of MS steady-state protein from isolated megagametophytes (●) and embryos (○) from various developmental stages was determined by scanning densitometry of SDS-PAGE immunoblots probed with immuno-affinity purified MS IgGs. Each data point shown is the mean of two determinations $\pm$ SD. Shown in the insert is a representative megagametophyte immunoblot probed with immuno-affinity purified MS IgGs. 20 μ g of buffer-soluble protein was loaded in each lane. Due to the low levels of MS protein in the embryo, immunoreactive MS in immunoblots was only observable on original overdeveloped blots and not resolved with photography.



loblolly pine seeds, immuno-affinity purified MS IgGs were used to probe protein blots of mature, stratified, and post-imbibition at 30°C (2-14 DAI) megagametophyte and embryo cell-free extracts (Figure 3.40). The relative amounts of 62 kDa MS steady-state protein (in parentheses) were quantified by the slot blot technique; after immunodetection and staining, MS protein levels were determined by scanning densitometry using purified MS protein as a standard (data not shown; see 2.10). Similar to MS enzyme activity, the levels of MS steady-state protein was very low in megagametophytes isolated from the mature (0.11 $\mu\text{g seed part}^{-1}$) and stratified seed (0.17 $\mu\text{g seed part}^{-1}$), then increased about 15-fold after 10 DAI (2.85 $\mu\text{g seed part}^{-1}$) (Figure 3.40). Following this peak, MS steady-state protein levels began to decrease slowly with megagametophyte tissue senescence. In the embryo, MS steady-state protein was undetectable at all stages examined, except at around 4 DAI, where only very low amounts were detectable on blots.

3.7.3 CAT

CAT enzyme activity was assayed in isolated megagametophyte and embryo cell-free extracts taken from mature, stratified, and post-imbibition at 30°C loblolly pine seeds (Figure 3.41). CAT enzyme activity was present at low levels in the megagametophyte from the mature seed (10.45 units seed part⁻¹) and rose slightly following stratification (18.18 units seed part⁻¹). CAT activity increased steadily following radicle emergence (4 DAI), peaking at 8 DAI (180.0 units seed part⁻¹), and then decreased sharply with megagametophyte tissue senescence (21.03 units seed part⁻¹ at 14 DAI). In embryos, CAT enzyme activity at all stages examined was comparably much lower than the CAT activity levels found in the megagametophyte (Figure 3.41). Activity of the enzyme was detectable at low levels in embryo cell-free extracts from mature (3.39 units seed part⁻¹) and stratified (5.94 units seed part⁻¹) seeds. CAT enzyme activity levels then rose slightly during germinative growth, increased to higher levels during post-germinative growth, peaking at around 10 DAI (30.67 units seed part⁻¹), and then staying relatively constant. This biphasic increase in CAT enzyme activity likely reflects the

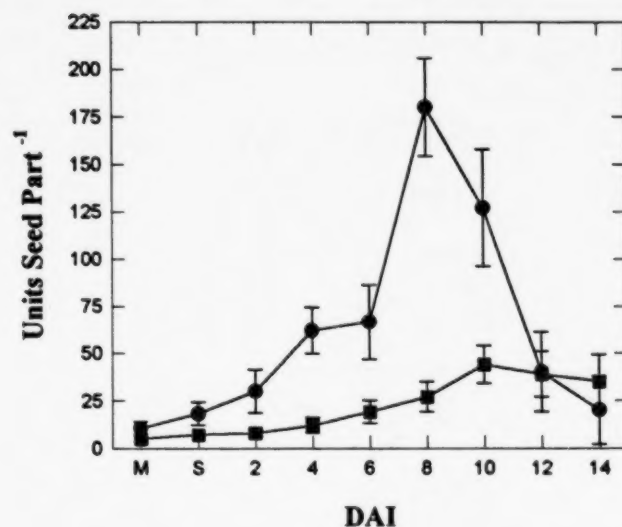


FIGURE 3.41. Profile of CAT enzyme activity in megagametophytes and embryos isolated from seeds through stratification, germination, and post-germinative growth. Cell-free extracts from mature (M), stratified (S), and following imbibition at 30°C (2-14 DAI) isolated megagametophytes (●) and embryos (■) were assayed for CAT enzyme activity. Each data point is the mean of three determinations $\pm$ SD.

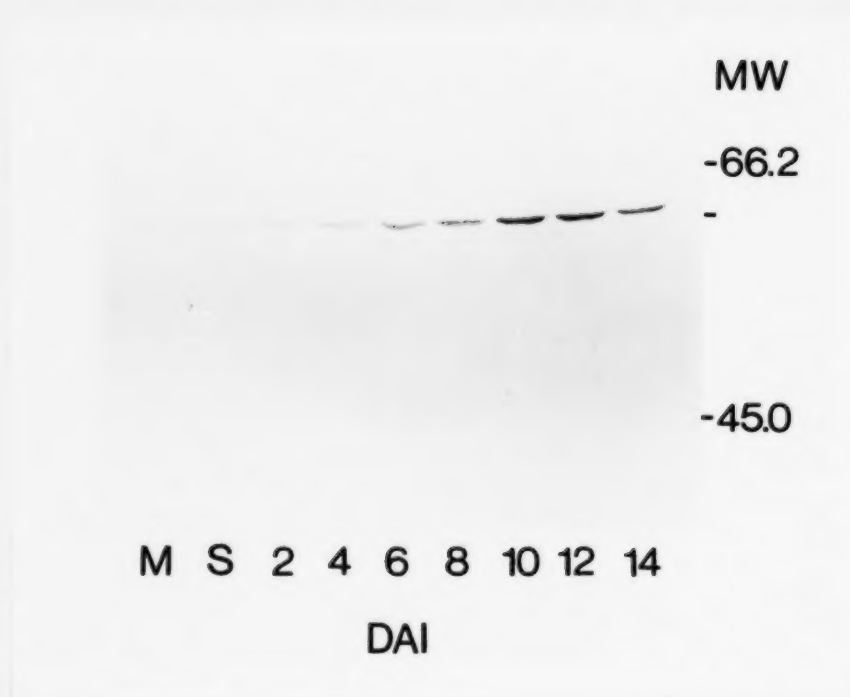


FIGURE 3.42. Immunoblot of CAT steady-state protein levels in megagametophytes following seed imbibition. Buffer-soluble protein extracts from megagametophytes isolated from mature (M), stratified (S), and 2 through 14 DAI seeds were subjected to 12% SDS-PAGE, electroblotted to nitrocellulose, and probed with CAT antiserum. For all lanes 20 μ g of protein was loaded. Numerical values to the right of the blot are the molecular mass (in kDa) or protein standards

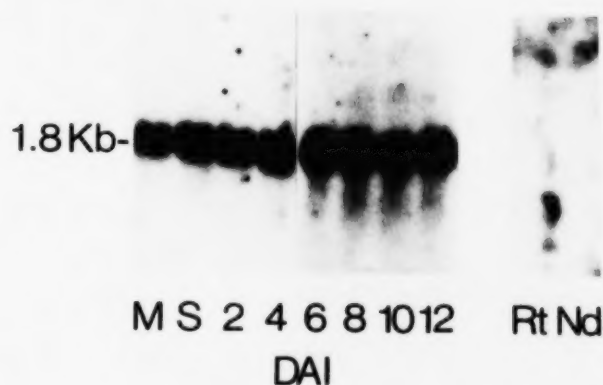


FIGURE 3.43. Accumulation of CAT mRNA in megagametophytes following seed imbibition. 10 μ g of total RNA isolated from megagametophytes from mature (M), stratified (S), and 2 through 12 DAI seeds, or roots (Rt) and needles (Nd) from a 4 month old loblolly pine tree, were separated on formaldehyde-agarose gel and transferred to nylon membrane. Blots were hybridized with a α [^{32} P]-radiolabelled cDNA probe encoding PtGEM 5-*Car0*, washed and exposed to X-ray film for 6 h at -70°C with intensifying screens. To assure relative uniformity in RNA gel loading, an identical gel was stained with ethidium bromide (data not shown).

transition from lipid degradation during germination to when the embryo is green and presumably photoautotrophic.

The levels of CAT steady-state protein in megagametophytes following seed imbibition were next examined. Shown in Figure 3.42 is an immunoblot of megagametophyte cell-free extracts from mature, stratified, and from seeds following imbibition at 30°C subjected to SDS-PAGE, transferred to nitrocellulose, and probed with CAT antiserum. The relative amounts of 59 kDa CAT steady-state protein (in parentheses) were quantified by slot blotting (data not shown). In a correlated fashion to the temporal changes of CAT enzyme activity levels, CAT steady-state protein was low in the megagametophyte from the mature seed (0.4 $\mu\text{g seed part}^{-1}$), increased slightly during stratification (1.3 $\mu\text{g seed part}^{-1}$), and then increased dramatically following seed germination (Figure 3.42). Accumulation of CAT steady-state protein during post-germinative growth peaked at around 10 DAI (25.7 $\mu\text{g seed part}^{-1}$), and then stayed relatively constant with only a slight decrease by 12 DAI (22.6 $\mu\text{g seed part}^{-1}$). In embryos, and although at very low levels, the relative accumulation of CAT steady-state protein following seed imbibition followed the same temporal pattern observed in the megagametophyte (data not shown). However, and due to these low levels and our limits of detection, CAT protein on immunoblots of embryo extracts were not resolved with photography, nor quantified by scanning densitometry.

The accumulation of CAT transcripts in megagametophytes following seed imbibition was examined by RNA gel and slot blotting. Total RNA, extracted from megagametophytes isolated from mature, stratified, and seeds following imbibition 30°C, was separated by formaldehyde-agarose gel electrophoresis, passively transferred to nylon membrane, and probed with PtGEM 5-*Cat0*. As shown in Figure 3.43, PtGEM 5-*Cat0* hybridized to a single RNA transcript of approximately ca. 2.0 kB in size. The relative levels of CAT transcripts were compared by way of slot blot analysis (in parentheses) and scanning densitometry (data not shown). CAT transcripts were prevalent in the megagametophyte of the mature seed, then increased 8-fold during stratification and 64-fold by the completion of germination (4 DAI). CAT transcripts continued to increase during post-germinative growth, peaking at around 8 DAI (114-fold), then decreased

slightly by 12 DAI. In roots and needles of 4 month loblolly pine seedling no CAT mRNA was detectable (Figure 3.43, lanes Rt and Nd). In general, the temporal accumulation of CAT transcripts in the megagametophyte following seed imbibition slightly preceded a parallel accumulation of corresponding CAT protein and enzyme activity.

3.7.4 ICL

The relative levels of ICL enzyme activity, protein, and mRNA in loblolly pine seeds following imbibition was examined. In the megagametophyte cell-free extracts, ICL enzyme activity was undetectable in the mature and stratified seed, then increased dramatically between the completion of germination (1.12 units per seed⁻¹, 4 DAI) and 8 DAI (14.81 units seed part⁻¹) (Figure 3.44). ICL enzyme activity then decreased dramatically with megagametophyte tissue senescence, with only low levels of enzyme detectable by 14 DAI (0.71 units seed part⁻¹). In the embryo, and similar to MS and CAT, the temporal accumulation of ICL activity was comparably lower than that of the megagametophyte (Figure 3.44). ICL enzyme activity was only detectable by around 2 DAI (0.08 units seed part⁻¹), and then began to increase during post-germinative growth, remaining relatively constant by 12-14 DAI (approximately 3.74 units seed part⁻¹).

The accumulation of ICL steady-state protein levels followed the same coordinated pattern as that observed for MS and CAT protein. Shown in Figure 3.45 is an immunoblot of megagametophyte cell-free extracts from mature, stratified, and seeds following imbibition at 30°C, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with ICL antiserum. Again, the relative amounts of ICL steady-state protein (in parentheses) were quantified by slot blotting (data not shown). ICL steady-state protein levels were low in the mature (0.2 µg seed part⁻¹) and stratified (0.4 µg seed part⁻¹) seed, and increased slightly during germination (3.2 µg seed part⁻¹, 4 DAI). Following germination, ICL steady-state protein increased dramatically, peaking around 10 DAI (14.4 µg seed part⁻¹) and then declined (Figure 3.45). In the embryo, and again similar to

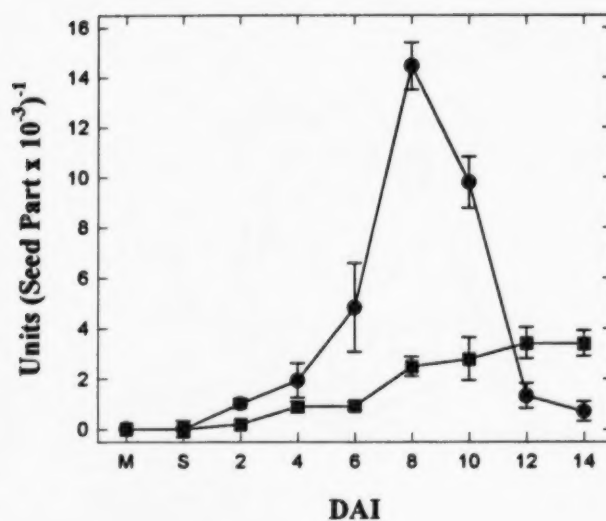


FIGURE 3.44. Profile of ICL enzyme activity in megagametophytes and embryos isolated from seeds through stratification, germination, and post-germinative growth. Cell-free extracts from mature (M), stratified (S), and following imbibition at 30°C (2-14 DAI) isolated megagametophytes (●) and embryos (■) were assayed for MS enzyme activity. Each data point is the mean of three determinations $\pm$ SD.

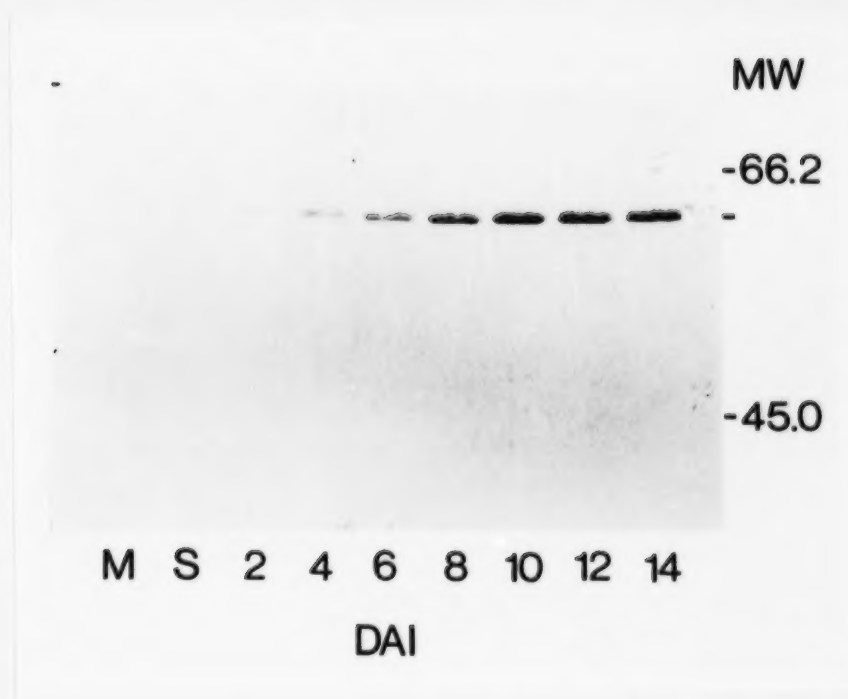


FIGURE 3.45. Immunoblot of ICL steady-state protein levels in megagametophytes following seed imbibition. Buffer-soluble protein extracts from megagametophytes isolated from mature (M), stratified (S), and 2 through 14 DAI seeds were subjected to 12% SDS-PAGE, electroblotted to nitrocellulose, and probed with ICL antiserum. For all lanes 20 μ g of protein was loaded. Numerical values to the right of the blot are the molecular mass (in kDa) of protein standards.

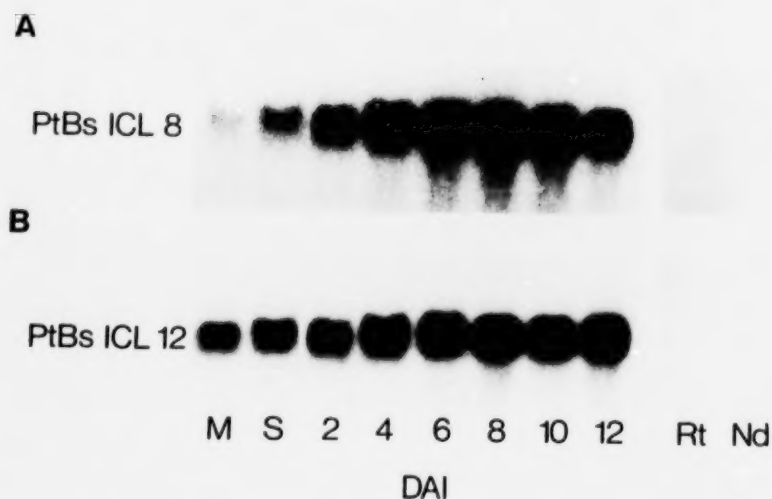


FIGURE 3.46. Accumulation of ICL mRNA in megagametophytes following seed imbibition. 10 μ g of total RNA isolated from megagametophytes from mature (M), stratified (S), and 2 through 12 DAI seeds, or roots (Rt) and needles (Nd) from a 4 month old loblolly pine tree, were separated on formaldehyde-agarose gel and transferred to nylon membrane. Blots were hybridized successively with a α [^{32} P]-radiolabelled cDNA probe encoding PtBs ICL 8 (A), stripped, and then re-probed with PtBs ICL 12 (B). Hybridization was performed at high-stringency conditions to avoid heterologous hybridization between the two cDNAs. Blots were exposed to X-ray film for 1 h (A) and 1.4 h (B) at -70°C with intensifying screens. To assure relative uniformity in RNA gel loading an identical gel was stained with ethidium bromide (data not shown).

MS and CAT, ICL steady-state protein levels on immunoblots were comparably lower than that observed in the megagametophyte. However, the protein did accumulate in a similar temporal fashion to the megagametophyte, peaking slightly at around 9 DAI (data not shown).

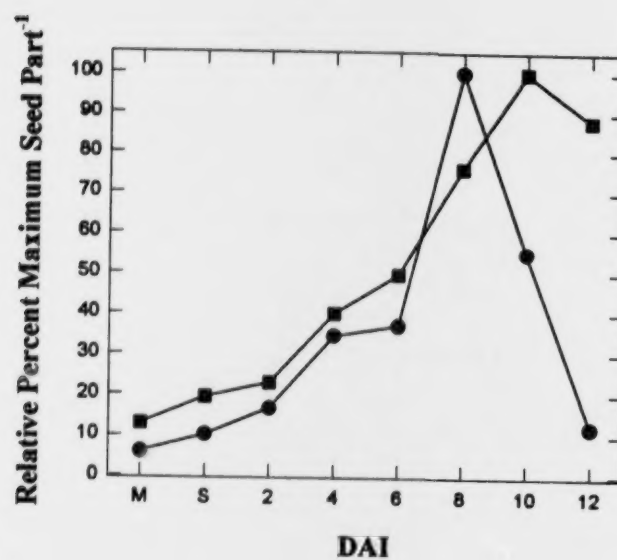
RNA blot and slot blot scanning densitometry analysis indicated that transcripts of both PtBs ICL 8 and PtBs ICL 12 accumulate during germination and post-germinative growth in loblolly pine seed megagametophytes. Shown in Figure 3.46 is a RNA blot of megagametophyte total RNA extracts from mature, stratified, and seeds following imbibition at 30°C. RNA was separated on a formaldehyde-agarose gel, transferred to nylon membrane and successively hybridized under stringent conditions with cDNA probes encoding PtBs ICL 8 (Figure 3.46 A) and PtBs ICL 12 (Figure 3.46 B). The accumulation of PtBs ICL 8 (ca. 2.0 kB) and PtBs ICL 12 (ca. 2.1 kB) transcripts occurred in a coordinate fashion in the megagametophyte following seed imbibition. However, PtBs ICL 12 transcripts were much more prevalent during seed stratification and germination. PtBs ICL 8 transcripts were present at low levels in the mature seed, increased slightly during stratification, then increased approximately 64-fold by the completion of germination (4 DAI) (Figure 3.46 A). In comparison, PtBs ICL 12 transcripts were present at much higher levels in the mature and stratified seed, yet increased to a similar 69-fold level by the completion of germination (Figure 3.46 B). Both PtBs ICL 8 and PtBs ICL 12 transcripts continued to accumulate during post-germination in the megagametophyte, peaking at around 8 DAI (121 and 131-fold, respectively). In roots and needles of 4 month loblolly pine seedling no PtBs ICL 8 or PtBs ICL 12 mRNA was detectable (Figure 3.46, lanes Rt and Nd).

3.7.4 *de novo* CAT and ICL Protein Synthesis

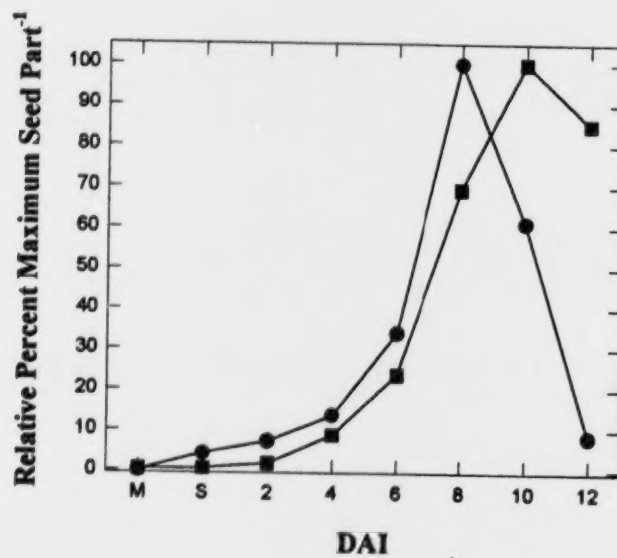
As illustrated in the previous sections (3.7.1-3.7.3) changes in enzyme activity and protein levels for CAT, ICL and MS occurred in a correlated fashion

FIGURE 3.47. Relative percent of maximum levels of CAT and ICL enzyme activity and protein following seed imbibition. Cell-free extracts of isolated megagametophytes from mature (M), stratified (S), and 2 through 12 DAI were either assayed for CAT and ICL enzyme activity (●), or subjected to protein blotting, probed with CAT or ICL antiserum, and levels of immunoreactive protein (■) determined by scanning densitometry. The relative amounts of CAT (A) or ICL (B) enzyme activity and protein levels shown here as a percent of maximum were determined by plotting individual values as a percentage of the highest value obtained (100%).

A.



B.



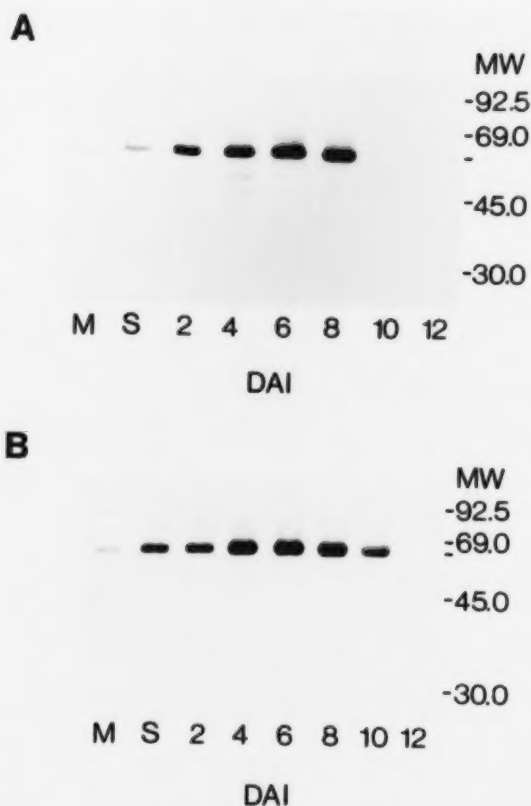


FIGURE 3.48. *De novo* protein synthesis of CAT and ICL protein following seed imbibition. Isolated megagametophytes from mature (M), stratified (S), and 2 through 12 DAI were labelled with [^{35} S]-methionine for 3 h. For all stages, CAT (A) or ICL (B) was immunoselected with CAT or ICL antiserum, respectively, from total buffer-soluble protein extracts diluted to contain equal counts (300,000 dpm). Immunoselected CAT and ICL was then subjected to 12% SDS-PAGE and fluorography. Indicated to the right of the fluorograph is the molecular mass (in kDa) of protein standards.

following imbibition at 30°C. However, during the later stages of megagametophyte post-germinative growth, the specific activities (units of activity per μg of protein) declined dramatically for all three enzymes. Shown in Figure 3.47 is the percent of maximum of CAT (A) and ICL (B) enzyme activity and steady-state protein accumulation in megagametophytes isolated from the mature, stratified, and seeds following imbibition at 30°C. Both CAT and ICL enzyme activity and protein levels increased in a coordinated and correlated manner up until 8 DAI. Then at 10 DAI, CAT and ICL enzyme activities began to decline dramatically, and by 12 DAI only 10.9% and 9.1% of the maximum level of activity was present in the megagametophyte for CAT and ICL, respectively. However, during these same stages of megagametophyte post-germinative growth, steady-state protein levels remained relatively constant, only decreasing to 89.1% and 86.3% of the maximum level of protein by 12 DAI for CAT and ICL, respectively.

To begin to examine this discrepancy in CAT and ICL enzyme to steady-state protein ratios during the later stages of megagametophyte post-germinative growth, the relative levels of *de novo* protein synthesis of both enzymes was measured. Megagametophytes, isolated from mature, stratified, and seeds following imbibition at 30°C were labelled for 3 h with [^{35}S]-methionine at 30°C. Buffer-soluble proteins were extracted and incubated with CAT and ICL antiserum, and the resulting immunoprecipitated products were then analyzed by SDS-PAGE and fluorography. Both CAT (Figure 3.48 A) and ICL (Figure 3.48 B) protein was synthesized *de novo* in megagametophytes from mature and stratified seeds. In both cases, *de novo* protein synthesis achieved relatively high levels by 6-8 DAI. However, by 10 DAI the *de novo* synthesis of CAT protein was undetectable on fluorographs (Figure 3.48 A). For ICL, *de novo* protein synthesis decreased over 52% (determined by scanning densitometry) by 10 DAI, and was undetectable on fluorographs by 12 DAI (Figure 3.46 B). Interestingly, during these same later stages of megagametophyte post-germinative growth, the translational efficiencies of CAT and ICL mRNAs appears to be unaffected. CAT and ICL antiserum was able to immunoprecipitate similar amounts of CAT and ICL protein from *in vitro* translation products of 8 and 10 DAI poly(A)⁺ RNA (data not shown). Apparently, some form of translational or post-translational processing is affecting the

differential decline of CAT and ICL enzyme activity and protein levels during the later stages of megagametophyte post-germinative growth.

3.8. Loblolly Pine Glyoxysomal Enzyme Biogenesis

3.8.1 CAT, ICL, and MS are not Synthesized as Higher

Molecular Weight Precursors

With only a few exceptions (Yamaguchi et al., 1984; Gietl, 1990; Preisig-Müller and Kindl, 1993; Kleff et al., 1994; Kato et al., 1995) most peroxisomal proteins are synthesized at their final size, and posttranslationally imported into the organelle without proteolytic processing during, or following sequestration (de Hoop and Ab, 1992; Olsen and Harada, 1995). This implies that the signal for targeting proteins into the peroxisome resides on the mature polypeptide. To determine if glyoxysomal proteins from loblolly pine are similar in this regard, *in vivo* and *in vitro* translation reactions were incubated with CAT, ICL, and MS antisera and immunoprecipitated products were analyzed by SDS-PAGE and fluorography. As shown in Figure 3.49 CAT (panel A), ICL (panel B), and MS (panel C) synthesized *in vivo* (lane A for all) and *in vitro* (lane B for all) had the same electrophoretic mobility on SDS-PAGE gels as purified protein or immunoreactive CAT (59 kDa), ICL (63.5/64 kDa), and MS (62 kDa) from isolated glyoxysomal fractions (see Figures 3.7 lane F; 3.19 lane B; 3.23 lane C). These results suggest that CAT, ICL and MS are not cleaved upon import into glyoxysomes of loblolly pine seed megagametophytes. However, because of the limited resolution of this approach, removal of a few amino acids from the newly synthesized protein upon import into the glyoxysome cannot be ruled out.

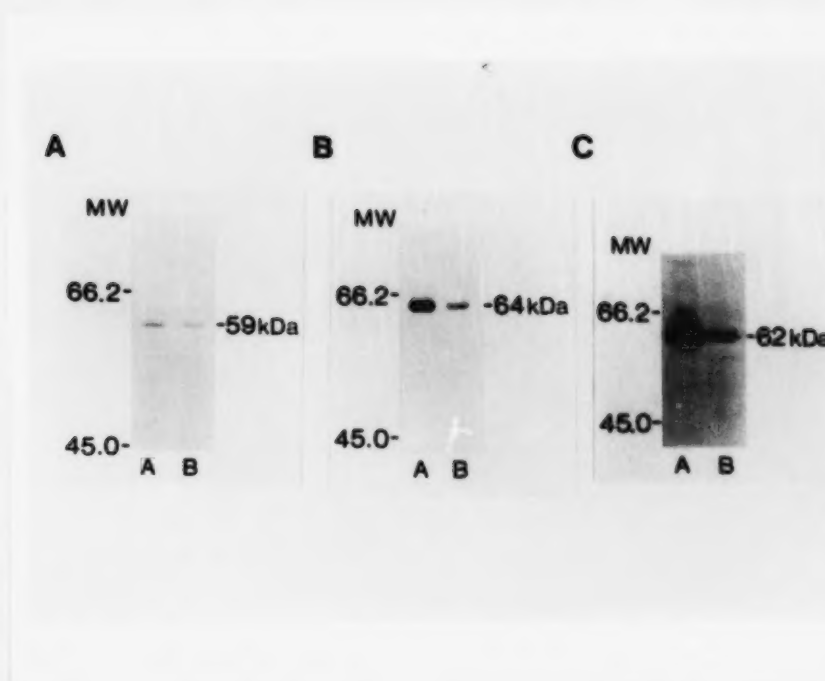


FIGURE 3.49. Comparison of *in vivo* and *in vitro* synthesized pine glyoxysomal proteins on SDS-PAGE and fluorography. CAT (A), ICL (B), and MS (C) were immunoselected from translation products of *in vivo* [^{35}S]-methionine labelled megagametophytes (8 DAI) following imbibition (lane A) and *in vitro* translated 8 DAI megagametophyte poly(A) $^+$ RNA (lane B) with pine CAT and ICL antiserum, or cotton MS antiserum. Immunoprecipitation products were then subjected to SDS-PAGE and fluorography. Indicated to the right of each fluorograph is the relative migration of the purified protein as determined by staining a portion of the gel with Coomassie blue R. Numerical values to the left of fluorographs are the molecular masses (in kDa) of protein standards.

3.8.2. Both Loblolly Pine ICLs Contain a Putative PTS at Their Carboxy Terminus

The prototype PTS is a carboxy-terminal tripeptide with a consensus amino acid sequence of SKL. Since Subramani and co-workers first demonstrated that this tripeptide functions as the PTS of firefly luciferase to the peroxisome (Gould et al., 1987) numerous other studies have demonstrated the extensive evolutionary conservation of this PTS among eukaryotes, including mammals, insects, plants, and yeast (Gould et al., 1990; Keller et al., 1991; Olsen and Harada, 1995). In plants, the SKL motif or a conserved variant thereof has been demonstrated to be sufficient and necessary for peroxisome import (Olsen et al., 1993; Trelease et al., 1994). With the availability of full-length cDNA clones encoding loblolly pine ICL, we examined the primary structure of the protein for any putative PTS's. Both PtBs ICL 8 and PtBs ICL 12 contain a carboxy-terminal tripeptide SRM or ARM, respectively, which are similar to other plant PTS reported for organelle import (Figure 3.50) (Olsen and Harada, 1995).

Interestingly, the C-terminal SKL tripeptide motif is also found in at least one other loblolly pine glyoxysomal matrix protein. Shown in Figure 3.51 is a 7% SDS-PAGE immunoblot of purified megagametophytic CAT, ICL, MS, and the photorespiratory enzyme glycolate oxidase, purified from 10 DAI loblolly pine cotyledons (Stone, Mullen and Gifford, unpublished), probed with antibodies raised against the SKL tripeptide. As expected the antiserum cross-reacted with both purified forms of ICL (i.e., ICL A 1, 64 kDa and ICL A 2, 63.5 kDa) (lane A). The SKL antiserum also recognized purified CAT protein (59 kDa, lane B) but did not cross-react with purified MS (62 kDa, lane C) or glycolate oxidase (43 kDa lane D) protein. Shown in lanes E and F are glyoxysomal and peroxisomal fractions isolated from 8 DAI megagametophytes or the dimorphic fungus *Yarrowia lipolytica*, respectively, as controls. A number of peroxisomal proteins from the fungus were immunoreactive (lane F), and at least one loblolly pine glyoxysomal protein with a similar molecular mass of ICL (64 kDa) cross-reacted with the SKL antiserum (lane E).

FIGURE 3.50. Comparison of deduced amino acid sequences of the carboxy-terminal regions of loblolly pine ICLs with other selected plant peroxisomal proteins. The predicted amino acid sequences of the C-terminal region of loblolly pine PtBs ICL 8 and PtBs ICL 12 was compared to castor bean ICL (*Ricinus communis*) (Beeching and Northcote, 1987), *Brassica napus* ICL (Comai et al., 1989), cotton ICL (*Gossypium hirsutum*) (Turley et al., 1990), cucumber ICL (*Cucumis sativus*) (Reynolds and Smith, 1995), *Brassica napus* MS (Comai et al., 1989), cucumber MS (Graham et al., 1989), castor bean MS (Rodriguez et al., 1990), cotton MS (Turley et al., 1990), spinach glycolate oxidase (Vолоkita and Sommerville, 1987), lentil glycolate oxidase (Ludt and Kindl, 1990), cotton subunit 1 and subunit 2 (Ni et al., 1990; Ni and Trelease, 1991), *Arabidopsis* (Chevalier et al., 1992), pea (Isin and Allen, 1991), tomato (Drory and Woodson, 1992) and maize CAT 1 (Redinbaugh et al., 1988). Sequences were obtained from Genebank and comparison of deduced amino acid sequences was carried out using the PC/Gene Clustal similarity search program. Gaps (dashes) have been introduced to maximize alignment. Putative targeting sequences are indicated in italicized bold type. Those proteins in which the SKL motif has been directly examined as a putative targeting signal for organelle import are indicated with asterisks.

Isocitrate Lyase

Loblolly pine 8	S S T L A L E S N I G A G T V L A K S R M
Loblolly pine 12	W S S D G R A L D L T N H G V V A R A R M
Castor bean*	T R P G A M E M G S A G S E V V A K A R M
Cotton*	T R P G A G N I G S E G N L V V A K A R M
<i>Brassica napus</i> *	T R P G A A G M G E G T S L V V A K S R M
Cucumber	T R E G A V N L G E E G N V V V A K S R M

Malate Synthase

<i>Brassica napus</i>	L T L A V Y D H I V A H Y P I N - A S R L
Cucumber	L T L D A Y N Y I V I H H P R E - L S K L
Castor Bean	L T L D A Y N N I V I H Y P K G - S S R L
Cotton	L T L D A Y N Y I V I H H P K D V S S K L

Glycolate Oxidase

Spinach*	R S H I A A D W D G P - - S S R A V A R L
Lentil	R E H I V A D W D T P R I Q P R A L P R L

Catalase

Cotton SU 1	S Q A D K S V G Q K L A S L L N V R P S I
Cotton SU 2	S Q A D K S L G Q K I A S R L N V R P S I
<i>Arabidopsis</i>	S Q A D K S L G Q K L A S R L N V R P S I
Pea	S Q A D R S L G Q K L A S H L N M R P S I
Tomato	S Q A D K S C G Q K V A S R L T V K P T M
Maize CAT 1	S Q C D A A L G Q K L P S R L N L K P S M

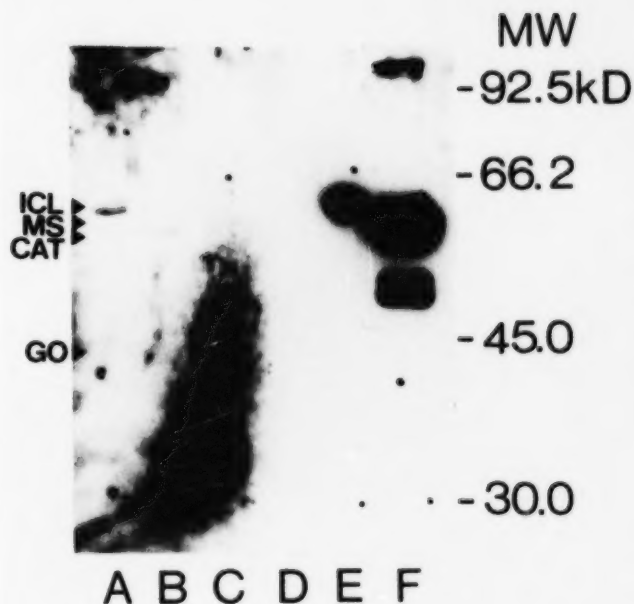


FIGURE 3.51. Immunoblot of purified loblolly pine glyoxysomal and peroxisomal proteins probed with SKL antiserum. Extracts of loblolly pine purified ICL (lane A), purified CAT (lane B), purified MS (lane C), purified glycolate oxidase (lane D), glyoxysomes isolated from 8 DAI megagametophytes (lane E), and peroxisomes isolated from the dimorphic fungus *Yarrowia lipolytica* were subjected to 7% SDS-PAGE, electroblotted to nitrocellulose, probed with SKL antiserum, and immunoreactive proteins detected by chemilluminescence staining. For lanes A-D 1.5 μ g of purified protein was loaded, and for lanes E and F 20 μ g of total protein was loaded per lane. The high background was the result of film overexposure due to the low titer of the SKL antiserum for pine extracts. Indicated to the left of the figure is the relative migration of purified ICL (64 and 63.5 kDa), MS (62 kDa), CAT (59 kDa), and glycolate oxidase (GO, 43 kDa). Numerical values to the right of the figure are the molecular masses (in kDa) or protein standards.

4. DISCUSSION

4.1. Purification and Properties of CAT

CAT has been purified and characterized from various bacterial, fungal, and animal sources (Schonbaum and Chance, 1976), and from a number of angiosperms, e.g. spinach leaves (Galston et al., 1951), cucumber cotyledons (Lamb et al., 1978), maize scutella (Chandlee et al., 1983), pumpkin cotyledons (Yamaguchi et al., 1986), sunflower cotyledons (Eising and Gerhardt, 1986), cotton cotyledons (Kunce et al., 1988), tobacco leaves (Havir and McHale, 1990), and castor bean endosperm (González et al., 1990, Ota et al., 1992; Mullen and Gifford, 1995). However, before the results presented in this thesis, CAT had never been reported purified from any gymnosperm source. The purification and subsequent availability of monospecific antibodies to the enzyme, indicated that loblolly pine CAT is very similar in the physicochemical properties reported for other higher plant CATs. For example, loblolly pine CAT is a tetrameric protein that is not glycosylated, and has a specific activity of 2215 units mg^{-1} .

CAT has been reported to exist in multiple forms in many higher plants, including spinach (Galston, 1955), mustard (Drumm and Shopfer, 1974), wheat (Singh and Singh, 1975), cotton (Kunce and Trelease, 1986), sunflower (Eising and Gerhardt, 1986), maize (Scandalios, 1987), soybean (Stegink et al., 1987), tobacco (Havir and McHale, 1989), and castor bean (Ota et al., 1992; Mullen and Gifford, 1995), with the numbers of isoforms varying from one in lentil cotyledons (Schiefer et al., 1976) to as many as twelve in mustard cotyledons (Drumm and Scopfer, 1974). In loblolly pine megagametophytes, CAT also exists as multiple forms. Here, CAT heterogeneity includes at least nine native isoelectric isoforms, and at least three charged protein subunits. In any study of isoform multiplicity it was important to demonstrate that the electrophoretic pattern of loblolly pine CAT isoforms was not the result of artifacts caused by limited proteolysis, enzyme aging, or electrophoretic conditions (Morikoff-Zwez et al., 1969; Holmes and Masters, 1972; Kunce and Trelease, 1986). The CAT isoforms shown in this thesis are real since

the same pattern was observed when extractions included either 20 mM 2-mercaptoethanol, 100 mM EDTA, or when gels were pre-electrophoresed, and when protease inhibitors such as leupeptin (1 mM), PMSF (2 mM) and/or benzamidine-HCL (1 mM) were included (data not shown). In fact, aberrations in the CAT isoform pattern were only observed when extractions were carried out in the absence of DTT, which resulted in the multiple isoelectric forms of loblolly pine CAT fusing to form one large, acidic smear. Interestingly, this phenomenon was not simply the result of proteolysis since the same sample subjected to SDS-PAGE and protein blot analysis revealed only a single 59 kDa immunoreactive band. Previously, Kunce and Trelease (1986) described the importance of the inclusion of DTT in all extraction buffers when examining the heterogeneity of CAT in cotton. Inclusion of DTT in extraction buffers was shown to stabilize CAT enzyme activity for at least 72 h and preserve the original CAT isoform pattern. As a result, Kunce and Trelease (1986) advised caution in any CAT isozyme analysis, since an anodic smear in the absence of DTT could be interpreted as a single electrophoretic band if less CAT activity was applied to the gel.

Although CAT heterogeneity has been identified in many plants, little is known about the molecular basis for this multiplicity. Only in cotton and maize have the biochemical and molecular aspects of CAT multiplicity been thoroughly examined (Scandalios, 1987; Ni and Trelease, 1991). Here, the five isoelectric isoforms are the result of two subunits in dynamic equilibrium with the tetrameric holoenzyme. Given the same interpretation of the subunit associations which occur in maize and cotton, the isoelectric heterogeneity of loblolly pine CAT is difficult to envision as the result of the associations of three charged subunits. Based on dynamic association and reassociation events between the three charged CAT subunits, this model would predict that twelve isoelectric forms of loblolly pine CAT would be resolved on non-denaturing gels, rather than the nine observed. Apparently, the classical CAT isozyme system of subunits associating equally to form charged isoelectric isoforms cannot be as easily applied for the mechanism of CAT multiplicity in loblolly pine. Nevertheless, in sunflower cotyledons another mechanism of CAT biogenesis exists that may be more applicable. Here, eight CAT isoforms (CAT 1-CAT 8) are formed through combinations of 59, 56, and 55 kDa

subunits (Eising et al., 1990). As a result of proteolytic processing, CAT 1 through CAT 5 are hybrids of 55 and 59 kDa subunits, whereas CAT 6 through CAT 8 contain 55 kDa subunits exclusively. In total, the number and abundance of CAT isoforms is the result of the selective assembly of specific subunits into unique tetrameric forms. Interestingly, this mechanism of a non-random, selective association of subunits, has also been suggested for CAT multiplicity in pumpkin (Yamaguchi et al., 1984), maize (Skadsen and Scandalios, 1986), potato (Sakajo and Asahi, 1986), and castor bean (Ota et al., 1992; Mullen and Gifford, 1995). In loblolly pine megagametophytes, only a single 59 kDa CAT subunit was resolved on 7% SDS-PAGE. Apparently CAT heterogeneity in loblolly pine does not involve the proteolytic processing that was reported for sunflower CAT. However, the three charged CAT subunits resolved on urea denaturing gels of megagametophyte cell-free extracts, or purified CAT protein, might still be interacting in a specific, non-random fashion, to form the nine native CAT isoforms. Here, specific combinations of any of the three CAT subunits could lead to the formation of the nine isoelectric isoforms. Although this interpretation is premature, a number of simple electrophoretic techniques, such as isolating gel purified fragments of each isoelectric CAT isoform and examining their respective subunit compositions on urea denaturing gels, would provide a direct test of the validity of this hypothesis.

At present a clear interpretation of the mechanism of CAT multiplicity remains to be determined. Nevertheless, results presented in this thesis do show that the specific activities of CAT isoforms A through I undergo a sequential transition during stratification, germination, and post-germinative growth. Following seed imbibition, the relative levels of steady-state protein for CAT isoforms A through I in the megagametophyte remained fairly constant. However, in megagametophytes from mature and stratified seeds, and from megagametophytes isolated from seeds during early germination (2 DAI), isoforms F, G, H, and I had higher specific activities than those of isoforms A and B. Conversely, during post-germinative growth, the specific activity of isoforms A and B increased, whereas F through I declined to undetectable levels. In nearly all other CAT isozyme systems where a sequential shift in the temporal pattern of CAT isoforms has been identified, the plant sources examined have been an epigeal

dicotyledonous seedling. Here the pattern shift in CAT isoforms is physiologically related to the change in the metabolic pathways occurring in glyoxysomes and leaf-type peroxisomes. In loblolly pine, and similar to endospermic seeds such as castor bean, the megagametophyte is unfunctional. Following lipid reserve mobilization and the embryo's emergent photoautotrophic competence, the megagametophyte tissue eventually senesces. Clearly, the shift in the specific activities of CAT isoforms in the megagametophyte following seed imbibition cannot be attributed to a transition from heterotrophic to autotrophic growth.

The process of loblolly pine seed stratification may provide an alternative developmental cue for this sequential shift in the pattern of CAT isoforms. In cucumber (Omran, 1980) and maize leaves (Taylor et al., 1974), as well as avocado fruit (Sharon and Khan, 1979), CAT activity has been reported to be involved in the dismutation of toxic H_2O_2 produced during a chilling treatment of $0^\circ C$. Recently, evidence has been presented that H_2O_2 may additionally have a signaling function in the cell. Prasad et al. (1994) have shown that a transient accumulation of H_2O_2 during chilling at $4^\circ C$ in maize seedlings induces a number of antioxidant enzymes, including CAT, which protect the cell from this enhanced H_2O_2 production. During seed stratification, loblolly pine seeds are exposed to temperatures of approximately $2^\circ C$ for up to 60 d. Although no increase in H_2O_2 levels have been reported during seed stratification, the biochemical responses known to accompany this chilling period include changes in growth regulators (i.e., auxin, gibberellic acid, and jasmonic acid), increases in nucleic acids, and modifications to respiration (Taylor et al., 1993 and references therein). Given the complex nature of the physiological changes and responses that occur during seed stratification, it is not unreasonable to speculate that an increase or alteration in H_2O_2 levels might be occurring in the megagametophyte during this period. If so, perhaps the high specific activity of isoforms F through I during stratification provides a protective function against oxidative stress and any damaging H_2O_2 that may be produced during this chilling period. Subsequently, during germination and post-germinative growth, the decline in the specific activities of isoforms F through I occurs concomitantly with an increase in the specific activities of isoforms A and B. Presumably, the accumulation of isoforms A and B are in

concert with the production of large amounts of H_2O_2 via acyl-CoA oxidase activity and the β -oxidation of fatty acids. At present, the applicability of this model remains to be determined, however it is apparent that CAT multiplicity and the developmental expression of CAT isoforms in loblolly pine megagametophytes is orchestrated by a seemingly complex mechanism(s).

4.2. Purification and Properties of ICL

Although ICL has been isolated and studied in a wide variety of plant sources, only in a few species, including only one gymnosperm source (Pinzauti et al., 1986), have homogeneous preparations been obtained (see Giachetti et al., 1987 and Vanni et al., 1990 for reviews). This is due to a number of problems encountered during the purification of the enzyme, such as the persistent contamination by CAT (Lamb et al., 1978; Frevert and Kindl, 1978; Pinzauti et al., 1982; Khan et al., 1992), charge heterogeneity (Lamb et al., 1978; Frevert and Kindl, 1978; Giachetti et al., 1982; Jameel et al., 1984; Pinzauti et al., 1986; Polanowski and Obendorf, 1991) and the intrinsic instability of the enzyme (Giachetti et al., 1982). Some of these same difficulties were experienced during the purification of ICL from loblolly pine megagametophytes. For example, only chromatography on HTP and octyl-Sepharose CL-4B provided the complete resolution of ICL from contaminating CAT (see section 4.4). Furthermore, and as discussed below, loblolly pine ICL possesses charge heterogeneity; separation on DE-52 anion-exchange resolved at least two native isoforms of the enzyme (ICL A and ICL B). However, loblolly pine ICL does not appear as susceptible to proteolytic attack, nor does the enzyme seem to possess an intrinsic instability. This is unlike the lability of enzyme preparations from other plant sources. This supposition seems to be confirmed by the observation that the final recovery of the enzyme was 30% of the initial homogenate activity. This value is comparably higher than those previously reported for other plant ICL preparations, including flax (14%) (Khan et al., 1977), cucumber (17%) (Lamb et al., 1978), and soybean (12%) (Polanowski and Obendorf, 1991). In fact, the 30% final yield for loblolly

pine ICL is likely an underestimate of the effectiveness of the purification procedure since only those fractions with high ICL and low CAT enzyme activity were selected for subsequent purification steps. In loblolly pine megagametophytes, ICL was purified 50.6-fold, to a specific activity of 4.455 units mg^{-1} , and a final product that was free of any contaminating proteins as judged from silver-stained gels. Interestingly, this criterion for the final purity of the enzyme has not been applied previously. With all other ICL preparations, purity has been based on Coomassie blue-stained gels alone.

The presence of multiple forms of native ICL has been reported for a number of other higher plants (see Vanni et al., 1990 for review). Khan et al. (1977), Vanni et al. (1979), Pinzauti et al. (1982), Pinzauti et al., (1983) and more recently Polanowski and Obendorf (1991) all described the presence of two forms of ICL resolved by chromatography on a DEAE anion-exchange column. In each of the reports, one form of the enzyme did not bind to the DEAE matrix, whereas the other form only eluted from the column with an increasing salt gradient. Loblolly pine ICL appears to be similar in this regard. In loblolly pine, the ICL form that eluted with the flow-through represented nearly 90% of the total activity. This form was designated ICL A, and the minor bound form ICL B. Frequently, the presence of multiple ICL forms reported in purification of the enzyme from higher plants has been attributed to proteolytic modifications, or conditions related to the age of the tissue extracted. In flax, Khan et al. (1977, 1979) described two forms of ICL on DEAE cellulose that were the result of selective proteolysis by the action of a low molecular weight endopeptidase. In *Pinus pinea*, Pinzauti et al. (1983) demonstrated that ICL multiplicity was related to the stage of germination, and at least in part, to proteolytic modification. Pinzauti et al., (1983) described two distinct forms of ICL on DEAE-Sephacel (forms I and II) whose relative amounts varied in the presence or absence of the protease inhibitor PMSF and/or the stage of germination the tissue was extracted. In loblolly pine, it is unlikely that ICL heterogeneity on DE-52 cellulose is the result of the selective proteolytic modification of the enzyme during purification. First, other protease inhibitors besides PMSF (i.e., leupeptin, benzadidamine and EDTA) were included in all the buffers used during the enzyme isolation. Furthermore, chromatography on DE-52 cellulose even in the absence of these protease inhibitors

resulted in an identical separation of ICL A and ICL B with the same relative abundance in activity peaks for both forms. Finally, antiserum to purified ICL A did not recognize ICL B, indicating that the two forms are immunologically unrelated and, therefore, distinct. This latter observation is in contrast to that reported by Polanowski and Obendorf (1991) who described that the two forms of soybean ICL resolved on DEAE-cellulose were immunologically identical. At present, loblolly pine ICL B, which represents 10% of the activity of the megagametophyte, remains to be characterized. However, due to its low total activity and because ICL A antibodies cannot be used for any type of affinity technique, the purification of ICL B will likely prove difficult.

An examination of the subunit composition of purified ICL A indicated that the heterogeneity of the enzyme was further complicated. Gel filtration of purified ICL A on a calibrated Sepharose CL-6B column eluted as a single peak with an estimated native molecular mass of 270 kDa. When analyzed by 7% SDS-PAGE, Coomassie blue staining or protein blots probed with ICL A antiserum revealed two polypeptides of 64 and 63.5 kDa (ICL A 1 and ICL A 2). Furthermore, IEF/SDS-PAGE protein blots probed with ICL A antiserum revealed that ICL A 2 further separated into two charged subunit isoforms. Nonetheless, loblolly pine ICL A is a tetramer composed of four subunits. This is in good agreement with nearly all previously published values for other higher plants (Vanni et al., 1990). However, and with only one other exception reported to date (Reizman et al., 1980), loblolly pine ICL does not appear to consist of subunits all having an identical molecular mass and/or charge (Vanni et al., 1990). In cucumber, Reizman et al. (1980) identified two subunit forms of ICL that were synthesized *in vivo* (61.5 and 60 kDa) and after RNA translation *in vitro* (63 and 61.5 kDa). Reizman et al. (1980) concluded that the heterogeneity of cucumber ICL is possibly the result of both *in vivo* forms being synthesized as larger precursors, reflecting the processing of the enzyme with its subsequent transport into the glyoxysome. Although the idea of post-translational modification(s) during glyoxysome protein biogenesis is now known to be generally invalid, the relationship of cucumber *in vivo* and *in vitro* ICL forms remains to be determined. In loblolly pine, a similar, and yet somewhat more complicated scenario of ICL heterogeneity exists. It may be that ICL A exists as a heterotetramer composed of

ICL A 1 and the two charged forms of ICL A 2, all undergoing recombination events. Alternatively, ICL A 1 and the two charged forms of ICL A 2 may be subunits of three homotetrameric forms of ICL A.

Although a number of possibilities may exist, loblolly pine ICL A subunit heterogeneity was demonstrated not to be an artifact generated during the purification of the enzyme. Since both ICL A 1 and ICL A 2 were immunoreactive when probed with cotton ICL antiserum, heterogeneity was not the result of a contaminant protein which may have simply co-purified with ICL during the isolation procedure (data not shown). Similarly, the subunit composition of ICL A is not the result of extraction artifacts (i.e. selective proteolysis) since all extraction conditions included a variety of protease inhibitors. Inclusion or absence of either leupeptin, PMSF, benzamidine and/or EDTA always resulted in all subunits being visualized from partially purified ICL A preparations or protein blots of various cell-free extracts. Finally, and similar to the conclusion proposed by Riezman et al. (1980), it is possible that ICL A 1 might represent a larger precursor form of ICL A 2 that includes a glyoxysomal signal peptide (de Hoop and Ab, 1992; Olsen and Harada, 1995). However, this interpretation is unlikely. Both ICL A 1 and the two charged forms of ICL A 2 were detected on protein blots of isolated megagametophyte glyoxysomal fractions probed with ICL A antiserum. A comparison of the relative mobility of *in vitro* and *in vivo* translation products on SDS-PAGE did not reveal any differences, confirming that ICL is not synthesized as a larger molecular weight precursor. Furthermore, an analysis of the deduced amino acid sequence for cDNA clones encoding loblolly pine ICL revealed no identity to the amino acid terminal presequences which have been identified in a small number of other glyoxysomal proteins (Olsen and Harada, 1995). In total, the heterogeneity of ICL in the megagametophyte of loblolly pine is a complex association of at least three protein subunits which together comprise one of the two native forms of the enzyme. At present, it is known that this multiplicity is not an artifact generated *in vitro*; however, any further understanding of ICL subunit combination events remains to be elucidated. In the future, using purified ICL A subunits and *in vitro* association-dissociation reactions (Scandalios, 1979; 1980), the formation of any

intergenic hybrid tetramers could be examined with the objective of providing a possible explanation of ICL heterogeneity in loblolly pine.

4.3. Purification and Properties of MS

To date, MS has been purified from a number of angiosperm plant species (Yamamoto and Beevers, 1961; Servattaz et al., 1973; Köller and Kindl, 1977; Bowden and Lord, 1978; Trelease et al., 1987; Mori et al., 1988; Khan et al., 1993), however the enzyme has never been isolated from any gymnosperm source. Compared to CAT and ICL, and based on a procedure described by Trelease et al. (1987), the purification of MS from loblolly pine megagametophytes was relatively simple for a number of reasons. First, all chromatography was carried out at room temperature, and the same buffer (with some minor modifications) was used throughout. Finally, the enzyme did not bind to any of the chromatography matrices including DE-52 cellulose, HTP, and phenyl-Sepharose CL-4B.

Typically, purification procedures for MS from angiosperm sources specifically avoid ammonium sulfate fractionation because the enzyme is known to be an especially hydrophobic protein, associating very easily, depending on the conditions, into aggregate forms (Bowden and Lord, 1978; Kruse and Kindl, 1983a 1983b; Dommès and Northcote, 1985; Trelease et al., 1987). During the isolation of MS from loblolly pine megagametophytes, ammonium sulfate fractionation between 0 to 35% saturation provided a 4.5-fold purification, but resulted in over a 60% loss of yield. Apparently, this loss of yield could be attributed to possible aggregation of the enzyme, however the subsequent resolution of loblolly pine MS on Sepharose CL-6B gel filtration, tends to suggest otherwise. In cucumber, one or two species of MS were observed on Sepharose CL-6B depending on whether $MgCl_2$ and glyoxylate were included, or omitted from buffers, respectively (Köller and Kindl, 1977). In cotton, gel filtration of MS on Sephacryl S-300 yielded two peaks of activity; the first a 150S highly aggregated form of the enzyme, and a second peak with a sedimentation coefficient of approximately 20S (Trelease et al., 1987). Attempts to eliminate this aggregated 150S form of the enzyme with higher levels of Mg^{2+} , glyoxylate, and by desalting on Sephadex G-25 columns prior

to gel filtration on Sephacryl S-300 were all unsuccessful (Trelease et al., 1987). In contrast, loblolly pine MS always eluted as one peak during molecular sieving on Sepharose CL-6B. This behavior of the enzyme was observed regardless whether $MgCl_2$ and glyoxylate were included in the sieving buffer.

Differences between the physicochemical nature of loblolly pine MS and MS isolated from other plant sources were also evident by its behavior on phenyl-Sepharose CL-4B. Previously, Kruse and Kindl (1983a) and Trelease et al. (1987) were able to purify MS from cucumber and cotton cotyledons, respectively, due to the fact that the enzyme(s) bound strongly to this hydrophobic interaction matrix. Irrespective of the conditions used, loblolly pine MS did not bind to phenyl-Sepharose CL-4B, but the column still provided an effective means of obtaining the final purified enzyme preparation. Interestingly, the inability of loblolly pine MS to bind phenyl-Sepharose CL-4B, as well as HTP, whereas other plant MS enzymes are adsorbed under similar conditions, suggests that pine MS may be biochemically distinct.

The octameric nature of loblolly pine MS is similar to that reported previously for other plant MS enzymes. Based on standardized molecular sieving, loblolly pine native MS has a molecular mass of 520 kDa, and appears to be comprised of eight 62 kDa monomeric subunits. However, this conclusion of pine MS subunit composition should be viewed with caution. Whereas plant MS have typically been reported as octamers, in yeast and bacteria the enzymes are tetrameric and monomeric, respectively (Sundaram et al., 1980; Bruinenberg et al., 1989). However, the subunit molecular mass of all these MSs, are very similar, ranging from 58-64 kDa. Recently, Khan et al. (1993) concluded that on the basis of previous reports of plant MS aggregation behavior, as well as hydrophobicity and phospholipid affinity, the *in vitro* oligomeric structure of the plant enzyme may be quite dissimilar to that *in situ*. It may thus be questioned whether loblolly pine MS *in situ* really occurs as an octameric enzyme; it might well be that under the conditions employed during purification, or as a result of its reported tight association (aggregation), loblolly pine MS *in vitro* forms lower order aggregates giving rise to an octomeric structure.

Based on the observations that loblolly pine MS does not react with Schiff's stain, nor is it absorbed onto immobilized Con A, it was concluded that it is highly improbable that the enzyme contains covalently linked carbohydrate residues. However, a number of our preliminary studies suggested that loblolly pine MS might be a glycoprotein. First, deglycosylation studies using the Endoglycosidase F, rather than PNGase F, resulted in a mobility shift of 17 kDa (62 kDa to 45 kDa) for purified MS on SDS-PAGE (data not shown). Second, polyclonal antibodies raised from apparently homogeneous purified 62 kDa MS protein recognized a number of other polypeptides besides MS on immunoblots. Included was one dominant immunoreactive polypeptide with a molecular mass of 45 kDa. Interestingly, this 45 kDa species was developmentally regulated in loblolly pine megagametophytes in a similar fashion to that of the purified MS polypeptide (data not shown). Finally, immunoblots demonstrated that these same MS antibodies also recognized a number of known glycoproteins including horseradish peroxidase and ovalbumin (data not shown). Because of these initial observations, it was postulated that antibodies elicited to the purified protein might be cross-reacting with protein-carbohydrate epitopes on the native enzyme as well as to other polypeptides. Furthermore, the 45 kDa polypeptide may represent a pre-processed, deglycosylated precursor, of a final 62 kDa MS glyoxysomal enzyme. However, and in agreement with the current model of glyoxysome/peroxisome biogenesis and mechanisms of protein targeting to the organelle (Trelease, 1984; Kindl, 1992; Olsen and Harada, 1995), further studies eventually determined loblolly pine MS to be devoid of a detectable carbohydrate component. For example, although the 62 kDa MS was cross-reactive, when protein blots were probed with cotton MS antiserum, the proposed 45 kDa pre-glycosylated MS species was not immunodetectable (data not shown). Similarly, the 45 kDa species was also not immunoreactive when protein blots were probed with immuno-affinity purified 62 kDa MS IgGs. In fact, it was immunoprecipitation of MS from *in vivo* and *in vitro* translated products which provided the strongest evidence against loblolly pine MS glycosylation. If MS was indeed glycosylated, *in vitro* labeled MS would have an altered mobility on SDS-PAGE to that of the *in vivo* form, migrating as lower molecular mass species. However, both *in vivo* and *in vitro* synthesized MS have the same 62 kDa

molecular mass of the purified protein, or immunoreactive MS from blots of megagametophyte glyoxysome fractions.

Interestingly, conflicting results and/or interpretations of MS glycosylation have also been frequently reported for other plants. Initially, MS from castor bean endosperms (Mellor et al., 1978) and cucumber cotyledons (Riezman et al., 1980) was reported to be glycosylated. However, MS isolated from the same two plant species were later shown not to be glycosylated (Lord and Roberts, 1982; Kruse and Kindl, 1983a). The conflicting nature of the apparent glycosylation of plant MS was also supported by two other observations. First, a number of glyoxysome matrix proteins were shown to be radiolabelled when castor bean isolated glyoxysomal fractions, or whole seed endosperms, were incubated with D-[^{14}C]-glucosamine (Bergner and Tanner, 1981; González, 1986). Secondly, in some species, MS enzyme activity was localized in ER fractions collected from sucrose gradients following ultracentrifugation (Lord and Bowden, 1978; González, 1982; Kruse and Kindl, 1983a). Based on these observations, González (1986) postulated a model of glyoxysomal matrix protein biogenesis. Here, and since the capacity for core glycosylation is confined to the ER, glyoxysomal glycoproteins such as MS must be routed through the ER before being eventually transported to the glyoxysome. However, recent evidence does not support this model, nor the likelihood of MS being a glycoprotein. First, no glyoxysomal proteins, including MS, have been shown to possess the necessary signal peptides that would be required for transit through the ER (Rodríguez et al., 1987; Comai et al., 1989; Graham et al., 1989; Turley et al., 1990). In fact, it has now been well established that glyoxysome matrix proteins such as MS are synthesized on free polyribosomes in the cytoplasm and post-translationally transported into the organelle with no involvement of the ER (Olsen and Harada, 1995). Second, and unlike the early reports by Bergner and Tanner (1981) and González (1986), glycosylated forms of glyoxysome proteins are not routinely found, and such modifications would be expected for ER-derived proteins. Third, and possibly the most significant evidence against the model proposed by González and the glycosylation of MS, is the recent demonstration that the previously reported relationship between MS and the ER is a result of the enzyme's ability to easily aggregate (Chapman et al., 1989). Using a variety of extraction

conditions (i.e., various buffers), Chapman et al. (1989) demonstrated that cottonseed MS is only localized with the ER on sucrose gradients as a result of the fortuitous association of aggregated forms of the enzyme and the organelle. Evidence presented in this thesis tends to support this recent model of glyoxysome protein biogenesis, and the likelihood that loblolly pine MS is not a glycoprotein. However, and similar again to the history of conflicting results for the enzyme's apparent glycosylation, a number of observations for pine MS still remain unanswered. First, why did incubation of MS with Endoglycosidase F, and not PNGase F, result in the enzyme's mobility shift from 62 kDa to 45 kDa on SDS-PAGE, suggesting an apparent deglycosylation? Similarly, characterization of the 45 kDa polypeptide, which was immunoreactive with purified MS antiserum and found to be developmentally regulated in a similar fashion to MS, remains to be determined. Interestingly, Khan et al. (1993) recently reported the presence of 45 kDa polypeptide which co-purified with maize MS and which was antigenically cross-reactive. Following limited proteolysis studies, Khan and co-workers eventually concluded that the 45 kDa species was a degradation product of the 64 kDa maize MS subunit. In loblolly pine, it remains to be determined if a similar situation holds true.

4.4. Co-Contamination of Loblolly Pine Glyoxysomal Enzymes

For most previously reported purifications of ICL and MS from higher plant sources, obtaining a homogeneous enzyme preparation has proven difficult due to the contamination of co-purifying CAT (Servettaz et al., 1973; Koller and Kindl, 1977; Frevert and Kindl, 1978; Lamb et al., 1978; Pinzauti et al., 1982; Pinzauti et al., 1986; Khan et al., 1992; Khan et al., 1993). The purification of ICL or CAT from loblolly pine megagametophytes followed the same shortcomings; CAT or ICL co-purified during the first four steps of the isolation procedure of each enzyme. In fact, only chromatography on HTP and phenyl-Sepharose CL-4B, or octyl-Sepharose CL-4B, allowed for the complete separation of CAT and ICL. For the purification of ICL, the use of hydrophobic interaction chromatography on octyl-Sepharose CL-4B has been demonstrated previously (Pinzauti et al., 1986). The ability of this matrix to effectively resolve both enzymes is

simply due to ICL's reported high hydrophobicity as compared to CAT (Vanni et al., 1990). Loblolly pine CAT did not bind as strongly as ICL to this matrix, thereby allowing for their complete resolution and obtainment of a homogeneous preparation for each enzyme. For MS, the enzyme co-purified with CAT and ICL for the first four steps of purification. Finally, MS was resolved and subsequently purified from both contaminants, by its inability to be absorbed onto phenyl-Sepharose CL-4B.

Based on the numerous reports of the similarity in the behavior of CAT and ICL during salt fractionation and column chromatography, Lamb et al. (1978) suggested that the chemical and physical properties of each enzyme appeared to be quite similar. Interestingly, a number of recent studies have led to a more intriguing hypothesis. Specifically, the behavior of CAT and ICL on some chromatography matrices has been shown to differ depending on whether both enzymes were present. Khan and co-workers demonstrated that the presence of maize ICL in CAT fractions prevents the latter protein from being absorbed onto DEAE-cellulose, whereas without ICL present CAT is absorbed (Khan et al., 1992). These results suggested that the troublesome co-purification of CAT and ICL is the result of a firm association of both enzymes *in vitro* and possibly *in vivo*. Khan et al., (1992) argued that such an *in vivo* association between both enzymes in the glyoxysome of an oilseed would be advantageous. Here, where β -oxidation of fatty acids and the glyoxylate cycle are operating at maximal levels, H_2O_2 is produced as a result of acyl-Co A oxidase and $FADH_2$ oxidation. H_2O_2 is known to be an extremely toxic substance, and glyoxylate, the product of the ICL reaction, is readily oxidized to yield formate and carbon dioxide. Therefore, the presence of CAT in the immediate vicinity of ICL would expectedly protect the glyoxylate molecules from unwanted oxidation. Recently, Beeckmanns et al. (1994) have demonstrated that such a situation likely exists. Based on an affinity electrophoresis technique, Beeckmanns et al., (1994) observed a direct association of maize MS and ICL that was due to a specific recognition mechanism, and not merely the result of non-specific hydrophobic or surface-charge interactions. Beeckmanns and co-workers concluded that MS and ICL, and probably other enzymes of the lipid degradation pathway of the glyoxysome, such as CAT, are associated *in vivo* as multienzyme clusters or metabolons. Such associations between consecutive enzymes of

the pathway offers a number of physiological advantages (i.e., protection of labile intermediates by reducing their life-times, circumvention of diffusion problems in bulk phase, and higher metabolic flux) (Beeckmanns et al., 1994). In support of this model, over the last decade the importance of enzyme-enzyme interactions for the regulation of other metabolic pathways has been amply demonstrated (Wombacher, 1983; Srivastava and Bernhard, 1986, 1987; Srere, 1987; Keleti, 1989; Beeckmans et al., 1993). Furthermore, it has been calculated that the overall protein concentration in various subcellular compartments, including the glyoxysome, may be as high as 0.5 g mL^{-1} (Fulton, 1982; Srere, 1987). At present, in loblolly pine any understanding of the *in vivo* associations between ICL, MS, and CAT remains to be elucidated. However, since these three enzymes displayed similar associations during column chromatography as that reported for maize it is not unreasonable to expect that the same situation *in vivo* might be occurring.

4.5. Construction of Loblolly Pine Megagametophyte cDNA Library

To enhance our chances of isolating clones encoding glyoxysomal enzymes such as CAT, ICL, and MS, a cDNA library was constructed from poly(A)⁺ RNA isolated from 8 DAI megagametophytes where each of these enzymes was presumably synthesized *de novo*. Evidence for this synthesis came from preliminary studies that indicated that the relative levels of CAT, ICL, and MS enzyme activity and steady-state protein were all high at around 8 DAI. In angiosperms, a consensus has emerged from numerous studies that have shown that the temporal expression of glyoxysomal enzymes (e.g., CAT, ICL, and MS) is correlated with the accumulation of their mRNAs (Thomas, 1993). Given the same interpretation of a transcriptional and/or post-transcriptional regulation of gene expression, and the relatively high levels of CAT, ICL and MS enzyme and steady-state protein, it was postulated that transcripts levels should all be correspondingly high during these same stages in loblolly pine megagametophytes. In support of this supposition, high

levels of CAT and ICL immunoprecipitable protein were detected on fluorographs when poly(A)⁺ RNA used for cDNA synthesis was translated *in vitro* and incubated with CAT or ICL antiserum. In general, the construction of the loblolly pine cDNA libraries with the UNI-ZAP XR system proved worthwhile because it eventually led to a reliable and relatively easy isolation of different ICL cDNAs (see below).

4.6. Isolation of cDNAs encoding for ICL

Although ICL has been purified and its biochemical properties extensively examined in a number of plant sources (see Vanni et al., 1990 for review), cDNAs encoding the enzyme have only been isolated from relatively few angiosperms, including sunflower (Allen et al., 1988), castor bean (Beeching and Northcote, 1987), *Brassica napus* (Comai et al., 1989), and cotton (Turley et al., 1990). Furthermore, only in *Brassica napus* (Zhang et al., 1993) and cucumber (Reynolds and Smith, 1995) has the isolation and characterization of genomic clones been described. Before this thesis, the isolation of a ICL cDNA from any gymnosperm source had not been reported.

In *Brassica napus*, ICL is encoded by at least two gene subfamilies which are expressed during late embryogeny and post-germination. Using restriction mapping, nucleotide sequence, and DNA gel blot analysis, Zhang et al. (1993) identified approximately six ICL genes in the *Brassica napus* genome that could be divided into at least two subfamilies based on their divergence in 5' and 3' non-coding regions. Although the examination of the subfamilies of *Brassica* ICL was based on genomic clones, in loblolly pine, isolated ICL cDNAs also indicate that the enzyme is likely encoded by a family of genes which consist of at least two classes. Immunoscreening of a loblolly pine megagametophyte λ ZAP cDNA library resulted in the isolation of a number of ICL clones, which based on restriction mapping and partial sequence identity of the 5' and 3' non-coding regions, could be classified into at least two groups. Obviously, further analysis including DNA gel blotting experiments, as well as the eventual isolation and characterization of genomic clones, will provide a better picture of the ICL gene family in

loblolly pine. Nevertheless, the results for loblolly pine ICL gene multiplicity reported here should not be surprising for several reasons. First, the loblolly pine genome is among the largest of the plant kingdom, containing up to 4×10^{10} bp per haploid nucleus (Gerttula et al., 1992). In addition, the vast majority of the DNA in the genome is found as repeated sequence families. As the result of genome mapping studies, Gerttula et al., (1992) demonstrated that a significant number of protein coding genes in loblolly pine occur in complex gene families. Second, many highly abundant plant proteins are encoded by gene families, and in loblolly pine, ICL represents nearly 2% of the total extractable megagametophyte protein. Finally, given the complex nature of loblolly pine ICL protein heterogeneity discussed previously, it is almost predictable that the organization of ICL would possess a similar degree of multiplicity.

From the two classes of loblolly pine ICL genes identified, two cDNAs were selected for further study. In view of the numerous and widely distributed differences between the sequences of PtBs ICL 8 and PtBs ICL 12, the likelihood that these sequence variations may have arisen from cloning artifacts and/or individual variations between clones was highly improbable. The coding sequences of PtBs ICL 8 and PtBs ICL 12 are 73% identical at the nucleotide level. Furthermore the two cDNAs do not appear to be alleles since their respective 3' and 5' non-coding sequences exhibit less than 28 and 41% identity. By the same token, the deduced amino acid sequences of the polypeptides encoded by PtBs ICL 8 and PtBs ICL 12 show 79% identity and have computed masses of 64,684 and 64,279 daltons, respectively. Interestingly, both these values are similar to the molecular masses of 64 and 63.5 kDa, found for purified loblolly pine ICL subunits ICL A 1 and ICL A 2. It is possible that PtBs ICL 8 and PtBs ICL 12 are cDNAs which could encode for ICL A 1 and one of the two charged forms of ICL A 2, respectively. At present, *in vitro* transcription and translation of PtBs ICL 8 and PtBs ICL 12, along with immunoprecipitations with monospecific ICL antiserum for ICL A 1 and ICL A 2 polypeptides, are being carried out to address this possibility.

4.7. Isolation of Partial CAT cDNA

To date, four CAT isozyme cDNAs from tobacco (Willekens et al., 1994a), three from maize (Bethards et al., 1987; Redinbaugh et al., 1988), two from cottonseed (Ni and Trelease, 1991; Ni et al., 1990), and one each from sweet potato (Sakajo et al., 1987), castor bean (González, 1993), pea (Isin and Allen, 1991), tomato (Drory and Woodson, 1992), rice (Mori et al., 1992), *Arabidopsis* (Chevalier et al., 1992), and mung bean (Mori and Imaseki, 1993) have been cloned, and their sequences determined either completely or partially (see von Ossowski et al., [1993] for a review). The nucleotide sequences of genomic clones encoding maize *Cat1* and *Cat3* (Abler and Scandalios, 1993; Guan and Scandalios, 1993), as well as two CAT genes from castor bean (Susuki et al., 1994) have also been examined. However, the isolation of a cDNA for CAT from any gymnosperm source has not been reported.

For loblolly pine, our initial strategy for isolating a full-length CAT cDNA was to simply screen our megagametophyte cDNA library with a radiolabelled heterologous probe. This same methodology has been the most frequent strategy employed by other laboratories for the isolation of CAT cDNA clones from angiosperm sources. However, in loblolly pine this same approach was unsuccessful. A primary screen of 3×10^5 recombinant phage from the megagametophyte library with a full-length cotton CAT cDNA only resulted in the identification of four positive plaques (data not shown). Furthermore, the signal intensity for each 'positive' was generally weak, and subsequent rounds of screening resulted in either a loss of a purified positive or inconsistent signal intensities. Attempts to alleviate these shortcomings, including further optimization of hybridization and washing conditions, or by using probes from smaller regions of the cotton CAT cDNA, all proved futile. Based on these observations it was postulated that the identity between loblolly pine and cotton CAT must be significantly divergent not to allow the isolation of a pine CAT cDNA with a heterologous probe. Interestingly these same troublesome observations were also demonstrated by RNA blot analysis. RNA blots of 3 DAI castor bean endosperm RNA and 8 DAI loblolly pine megagametophyte total

RNA or poly(A)⁺ RNA, were probed with a radiolabelled cotton CAT full-length cDNA. In all cases, with equal exposure times, the cotton cDNA probe easily recognized a 2.1 kB castor bean CAT transcript (González et al., 1993; Mullen and Gifford, 1995). However, no detectable transcripts were observed for loblolly pine (data not shown). In fact, only when blots were hybridized and washed under atypically low stringency conditions, and exposed for several days, as opposed to hours, was a faintly detectable loblolly pine CAT transcript observed (data not shown). Interestingly this apparent lack of identity, or at least the difficulty of using angiosperm heterologous probes with loblolly pine RNA, is not unique for CAT. Similar results have also been observed using cDNAs for cotton ICL and MS, *Brassica* MS, spinach glycolate oxidase, and a clone encoding a cotton LEA gene, as well as a *Brassica* MS cDNA as a probe with Douglas-fir RNA (S. Misra, personal communication). Reverse blotting experiments of angiosperm RNA with pine cDNAs (i.e. ICL or CAT) as radiolabelled probes also failed. In addition, protein blotting experiments using loblolly pine and angiosperm polyclonal antibodies also provided evidence for this lack of similarity. Although cross-reactivity of proteins from different species to polyclonal antibodies is not uncommon, studies in this thesis extend the observation for a lack of identity between gymnosperm and angiosperm glyoxysomal proteins. In all cases, loblolly pine CAT, ICL, and MS antiserum cross-reacted with a number of other gymnosperms including lodgepole pine, white spruce and Douglas fir. However, castor bean and cotton extracts separated on the same blots displayed a markedly reduced serological cross-reactivity. Similarly, and in a reverse fashion, cotton CAT, ICL, and MS antiserum cross-reacted with castor bean protein extracts more intensely than any of the gymnosperms examined (data not shown). These observations were not completely expected given the high degree of identity between the deduced amino acid sequences of loblolly pine CAT and ICL, and cDNAs encoding these proteins from cotton and castor bean. In total, the results presented here, as well as those observed by other laboratories examining gene expression in gymnosperms, suggests the need for precautions when assessing the validity of angiosperm cDNAs, or antiserum, as tools for heterologous studies.

The next strategy for isolating a full-length cDNA clone encoding CAT was to use PCR. Here, degenerate primers based on conserved domains of plant CATs were synthesized. Combinations of these primers during PCR using cDNA isolated from the 8 DAI loblolly pine megagametophyte library as a template were designed to produce two products; the 5' and 3' halves of the protein coding region of CAT. The resulting product from the 5' half of the protein coding region was amplified, and this fragment was then subcloned and sequenced to entirety. However, and even by varying PCR conditions for optimization, a product from the 3' reaction was not obtained. Conceivably, and similar to the explanation for the failure of a heterologous probe, the reason for no PCR product in this reaction may be due to the overall lack of identity in the 3' half of the loblolly pine cDNA compared to known angiosperm CAT cDNAs. Recently, González (1991) showed that although the overall amino acid sequence identity of plant CATs is high, ranging from 68 to 95%, only 33% of the amino acids within the carboxy terminal of the protein are conserved. In loblolly pine, CAT exhibits an overall identity of 85% and 74% to its angiosperm counterparts at the amino acid and nucleotide level, respectively. Therefore, and based on observations by González (1991), it appears likely that there could be an increase in the variability of the sequence identity in the 3' half of the protein. Furthermore, this apparent variability of loblolly pine to angiosperm CAT sequences in the carboxy terminal may also occur in the small number of regions that were conserved in angiosperm CATs and used here for PCR primer synthesis.

Since the partial loblolly pine CAT cDNA was obtained by PCR amplification with degenerate primers based on conserved domains of angiosperm CATs, the formal possibility exists that cDNAs encoded by different genes could have served as PCR templates, yielding a hybrid cDNA. However this possibility appears highly unlikely. Nevertheless, the existence of multiple CAT subunits does suggest that loblolly pine CAT may be encoded by multiple genes and that a genetic analysis will be difficult. PCR amplification of CAT cDNAs never resulted in a CAT gene sequence that differed from that of pGEM 5-*Cat0*. Furthermore, RNA blot analysis provided no evidence of multiple transcripts among RNA populations from any developmental stage examined. In tobacco, four CAT cDNAs, with identities ranging from 87 to 93%, were isolated using a similar

PCR approach (Willekens et al., 1994a). However, Willekens et al., (1994a) cautioned that due to the high sequence similarity between CATs of one species, discrimination between isozymes during screening procedures may be particularly difficult. At present a full-length CAT cDNA or any of the other putative CAT cDNA isozymes from loblolly pine megagametophytes remain to be obtained. However, and facilitated by the availability of monospecific antibodies for each of the three charged protein subunits, and the sequence information of the 5' half of pGEM 5-*Cat0*, the isolation of CAT isozyme cDNAs should likely be possible.

CATs from dicotyledonous angiosperms can be grouped into at least three classes. Recently Willekens et al. (1994b) used DNA sequence analysis of tobacco CAT cDNAs compared to other dicotyledonous CATs to identify three structurally distinct classes of CATs based on a functional organization. One class consisted of cotton 2, *Arabidopsis*, tomato, and Cat1 from tobacco, all isolated from light-grown-leaf-specific libraries, indicating that this class of CAT are involved in photorespiration. For the second class, five CATs, including cotton 1, Cat3 from tobacco, *Glycine max*, pea, and *Vigna radiata* are all expressed in organs that contain glyoxysomes. Finally, and more distantly related to the first two groups, is another class of CAT represented by Cat2 from tobacco, and a CAT from sweet potato tubers. This third group of CAT appears to be associated with, or localized to, unspecialized microbodies, a class of peroxisomes that are abundant in roots and tubers. Based on a comparison of the 5' half of the protein coding regions, loblolly pine CAT is the most similar to cotton CAT 1 and the group of dicot CATs classified here as localized in organs containing glyoxysomes (von Ossowski et al., 1993; Willekens et al., 1994b). Obviously, a full-length CAT cDNA from loblolly pine will be required to make a more complete phylogenetic comparison. Nevertheless, this preliminary conclusion for loblolly pine CAT as related to CATs from dicotyledonous plants localized in glyoxysomes could be supported by the unifunctional physiological nature of the megagametophyte.

4.8. Changes in mRNA Populations following Seed Imbibition

In loblolly pine and other conifer seeds, stratification, germination and post-germinative seedling growth are characterized by the accumulation and disappearance of distinct *de novo* synthesized protein subsets (Stabel et al., 1990; Gifford et al., 1991; Groome et al., 1991; Schneider and Gifford, 1994). Groome et al. (1991) identified three categories of proteins in both the megagametophyte and embryo of germinating and post-germinative loblolly pine seeds. Included was one group of proteins which accumulate to high levels following radicle emergence and likely represents hydrolytic enzymes involved in protein and lipid reserve mobilization (Groome et al., 1991). During loblolly pine seed stratification, changes also occur in the *de novo* synthesis of proteins which appears to be related with increased seed germinability (Schneider and Gifford, 1994). Coinciding with the biochemical and physiological alterations which underlie seed stratification, germination, and post-germinative growth in loblolly pine seeds, were changes in the patterns of mRNA populations in both the megagametophyte and embryo. In some angiosperm seeds, similar types of studies have identified unique mRNA populations and gene sets which are expressed during seed development, germination, and post-germinative seedling growth (Dure et al. 1981; Galau and Dure, 1981; Laroche-Raynal et al., 1984; Misra and Bewley, 1985; Datta et al., 1987; Harada et al., 1988; Sanchez-Martinez et al., 1988; Parcy et al., 1994). In cotton cotyledons, for example, changing sets of mRNA populations have been linked to the major physiological changes occurring in the seed during these developmental periods (Dure et al. 1983; Galau et al. 1991).

The changes in mRNA populations during loblolly pine seed stratification, germination, and post-germination presented in this thesis are an extension of the previous examination of the overall changes in the patterns of *de novo* protein synthesis (Groome et al., 1991; Schneider and Gifford, 1994). These studies were designed to precede any experiments dealing with the developmental expression of specific germination and/or post-germination cDNAs (i.e. CAT and ICL) in hopes that they would provide convenient markers and a clear picture of the more global changes in gene expression which are occurring in loblolly pine seeds following imbibition.

In conifers, (Yamamoto, 1982; Whitmore, 1991; Masumori et al., 1992), as well as angiosperm seeds (reviewed in Payne 1976), certain mRNAs preformed during the later stages of seed maturation persist in the seed as stored mRNAs. These messages decay rapidly following the beginning of germination, and are believed to be involved, or essential for, early protein synthesis (Bewley and Marcus 1990; Bewley and Black 1994). Loblolly pine seeds appears to be similar in this regard. In both the megagametophyte and embryo of the mature dormant seed, messenger RNAs were able to direct the *in vitro* synthesis of a wide range of polypeptides with molecular masses between 15 and 100 kDa. In the megagametophyte, a number of messenger RNAs stored in mature seed disappeared rapidly during germination, and were undetectable following radical emergence. Interestingly, in angiosperms, specific examples of stored mRNAs which are degraded during seed germination are the late embryogenesis abundant (LEA) proteins. These proteins are believed to play a role in cellular protection during seed desiccation and dormancy (Dure et al., 1989). In conifer seeds, LEA proteins have yet to be identified. However, recently, Leas and Misra (1993) did report the cross-hybridization of a radish LEA cDNA to white spruce RNA isolated from various stages throughout seed development. White spruce transcripts accumulated prior to seed desiccation, and reached maximal levels in the mature seed in a similar temporal fashion as that reported for angiosperm LEA messages. In loblolly pine, the subset of messages which are stored in the mature seed and then rapidly decline during germination could possibly encoded LEA or LEA like proteins. However, this conclusion, as well as those reported by Leal and Misra (1993) are at best speculative. Nonetheless, it now remains to be determined if LEA proteins do exist in conifer seeds, and whether they have a similar functional role(s) during seed development as they do in angiosperm seeds.

Loblolly pine seeds exhibit an imposed dormancy, and require a moist chilling treatment, or stratification, at relatively low temperatures for several weeks to prompt seed germination (Biswas et al., 1973; Carpita et al., 1983). Previously, Whitmore (1991) reported no differences in the mRNA populations of mature and stratified eastern white pine seeds. Whitmore (1991) concluded that stratification appears to not effect transcription but rather the translational activity of select stored mRNA species. In

contrast, Murphy and Hammer (1993) and Taylor et al. (1993) both demonstrated quantitative changes in the *in vitro* synthesized translation products during seed stratification in sugar pine and Douglas fir, respectively. In particular, Taylor et al. (1993) identified a number of proteins whose synthesis increased during stratification in a manner concomitant with the increased germinability of the seed. In loblolly pine, at least two subsets of *in vitro* synthesized proteins display qualitative as well as quantitative changes during the 35 d stratification period. Previously, Schneider and Gifford (1994) identified a similar pattern in the *de novo* synthesis of a diverse set of proteins in the megagametophyte and embryo of loblolly pine seeds during stratification. Schneider and Gifford (1993) concluded that either the synthesis of some proteins, or the relative decline of others, could possibly account for the imposition and/or maintenance of embryo dormancy. The appearance and disappearance of mRNA populations shown in this thesis indicates that the changes in the *de novo* protein synthesis reported earlier (Schneider and Gifford, 1994) is likely the result of the differential expression of specific genes and not simply the translation of mature seed mRNAs during the stratification process. It has yet to be determined what roles these proteins play during the maintenance and loss of loblolly pine seed dormancy. In relation to this, in sugar pine, a number of enzymes such as a leucine-tyrosine aminopeptidase and sucrose synthase are synthesized during seed stratification, and are believed to play a role in the dormancy-breaking process (Murphy and Hammer, 1993).

Seed germination and early post-germinative growth are developmental processes that are marked by numerous biochemical and physiological alterations (Bewley and Marcus 1990). In loblolly pine seeds, these developmental stages are characterized by the hydrolysis and mobilization of protein and lipid storage reserves. Initially, these reserves are hydrolyzed slowly during germination, then rapidly following radicle emergence (Groome et al. 1991). Interestingly, these hydrolysis events are accompanied by the *de novo* synthesis of distinct protein subsets (Groome et al., 1991). In agreement with these previously reported developmental shifts in the patterns of *de novo* synthesized proteins, mRNA populations change in a similar temporal fashion. In the megagametophyte and embryo one mRNA subset decreased immediately following imbibition at 30°C,

disappearing at around radicle emergence. In the embryo, another mRNA subset was present only during germination. To date, the identity or characterization of genes encoding germination specific proteins remains limited. However, a general consensus for a role of these proteins has emerged whereby they are believed to be involved in the overall cellular regulation or increased metabolic state of the seed (Bewley and Marcus 1990; Bewley and Black 1994). In angiosperm seeds, one example of a protein newly synthesized during germination is the wheat homopentameric glycoprotein, germin. Recently, Lane et al. (1993) reported that germin is an oxalate oxidase, believed to play a possible role in H_2O_2 generation and cell wall restructuring in the germinating and growing wheat embryo. In conifers, it remains to be determined if similar proteins are involved in the germination process. However, the pattern of mRNA species during loblolly pine germination reported here does suggest that distinct gene sets are regulated in a modulated fashion during this developmental period.

In loblolly pine, accompanying the rapid hydrolysis of reserves during post-germinative growth is an increase in the *de novo* synthesis of a number of proteins (Groome et al. 1991) and an overall increase in the RNA content of the megagametophyte and embryo (King and Gifford, unpublished). In general, these increases are believed to be involved in the synthesis of messages encoding storage reserve hydrolytic enzymes (Gifford et al. 1991). In this thesis, post-germinative growth in loblolly pine is shown to be marked by a dramatic increase in the synthesis of a number of mRNA species. Messages, at low levels or undetectable during germination, increased rapidly following radicle emergence, and reached high levels by 8 days after imbibition at 30°C. In angiosperms, similar observations of the rapid accumulation of distinct mRNAs following seed germination have been reported (Dure et al., 1981; Misra and Bewley, 1985; Datta et al., 1987; Harada et al. 1988; Sanchez-Martinez et al., 1988). To date, much of our understanding of the regulation of genes encoding mRNAs which accumulate post-germination comes from studies of oilseeds and the hydrolytic enzymes CAT, ICL, and MS. Here, these glyoxysomal enzymes are regulated at the transcriptional level; their messages accumulate in a concomitant manner with the rates of lipid hydrolysis (Thomas, 1993). Given the same interpretation of the regulation of these enzymes as in angiosperm

oilseeds, then it is not unreasonable to speculate, that in loblolly pine some of the dramatic increases in the mRNA species shown here may include CAT, ICL and MS transcripts. In fact, the relative mRNA levels for two of these enzymes, CAT and ICL, do accumulate to high levels following germination in loblolly pine seeds.

4.9. Regulation of CAT, ICL and MS Gene Expression

In angiosperms, a consensus has emerged whereby the expression of glyoxysomal enzymes such as CAT, ICL, and MS in oilseeds is primarily transcriptional; peak mRNA accumulation occurs about 24 h before peak protein and/or enzyme activity (Weir et al., 1980; Smith and Leaver, 1986; Allen et al., 1988; Rodriguez et al., 1987; Comai et al., 1989; Ettinger and Harada, 1990; Redinbaugh et al., 1990; Turley and Trelease, 1990; Ni and Trelease, 1991; Thomas, 1993). In loblolly pine, although transcriptional regulation also appears to be the primary mechanism for the expression of glyoxysomal enzymes. A number of observations suggest that post-transcriptional processes also differentially affect the accumulation of CAT, ICL and MS steady-state protein and enzyme activities. For example, qualitative analysis indicated that CAT, ICL and MS enzyme activity and steady state protein levels were undetectable or low, respectively, in the mature seed, increased slightly during stratification and germination, and then increased dramatically during post-germinative growth, peaking at around 8-10 DAI. In a parallel manner, the levels of CAT and ICL mRNA also increased during stratification and germination, peaking at least 1-2 d before enzyme activity. However, when the regulation of CAT and ICL gene expression, as well as MS to a lesser extent, were compared on a quantitative basis as opposed to a qualitative one, a non-concerted development of mRNAs, steady-state protein, and enzyme activities was observed. For example, RNA blot analysis indicated that CAT transcripts as well as ICL mRNA hybridizing to PtBs ICL 8 and PtBs ICL 12 were all detectable at relatively high levels in the megagametophyte of the mature, stratified and germinating seed. These same stages no or very little enzyme activity, and only a small detectable amount of steady-state protein was present for both enzymes. In a similar fashion, the relative accumulation of protein and enzyme activity in *Brassica napus*

embryos and seedlings does not directly correspond to ICL and MS mRNA. Ettinger and Harada (1990) demonstrated that when qualitative increases in mRNA, protein, and enzyme activity were examined quantitatively, the seemingly transcriptional and co-ordinated regulation of ICL and MS was instead non-co-ordinated and other processes (post-translational ?) influence the levels of activity for both enzymes.

In loblolly pine, ICL and MS enzyme activity were not present in the megagametophyte of the mature seed. However, ICL and MS protein was immunodetectable on protein blots. This apparent lack of ICL and MS activity may not be the result of post-translational regulation but simply the presence of an enzyme inhibitor in megagametophyte extracts. In cucumber (Frevert et al., 1980), sunflower (Theimer, 1976; Fusseder and Theimer, 1984), and cotton (Choinski and Trelease, 1978), the difficulty of reliably measuring ICL activity in the mature seed has been attributed to an apparent inhibitor. In loblolly pine megagametophytes, the presence of an enzyme inhibitor for ICL and/or MS, remains to be determined. At present the only conclusion that can be made based on these results is that the discrepancies in specific activities of both ICL and MS in the mature seed indicates that some other form of regulation besides transcriptional is effecting enzyme activity.

Further evidence for a post-transcriptional, non-coordinated regulation of loblolly pine glyoxysomal enzyme gene expression was evident on RNA blots where PtBs ICL 8, PtBs ICL 12, and pGEM 5-*Car0* are expressed differentially. When examined qualitatively, CAT and ICL mRNA both increased following germination in a coordinate manner. However, quantification of these transcript levels, indicated that the relative levels of CAT mRNA in the megagametophyte of the mature seed were 24 and 11-fold higher than PtBs ICL 12 and PtBs ICL 8 mRNA, respectively. These higher levels of CAT transcripts in the mature and stratified seed may reflect the possibility that pGEM 5-*Car0* encodes a form of CAT that is expressed during the early stages following seed imbibition (i.e. stratification). Finally, when PtBs ICL 8 and PtBs ICL 12 transcripts were compared, PtBs ICL 12 mRNA levels were 7-fold higher in the megagametophyte of the mature seed. Following germination, the larger relative increase in PtBs ICL 8 subsequently resulted in mRNA levels that were 3-fold higher than PtBs ICL 12 at 8 DAI.

At present it is not known whether PtBs ICL 8 and PtBs ICL 12 corresponding encode the protein subunits ICL A 1 or ICL A 2. Therefore, a quantitative comparison of the relative accumulation of these transcripts to the levels of steady state protein cannot be made. Nevertheless, it is apparent that although CAT and ICL, and most likely MS, are regulated primarily at the transcriptional level in a non-coordinated fashion, some post-transcriptional processes appear to influence the accumulation of each enzyme.

The most typical example of a mechanism of post-transcriptional control of glyoxysomal enzymes operating in loblolly pine megagametophytes occurs during the later stages of post-germinative growth. At 10 DAI, CAT and ICL enzyme activities were both rapidly declining, and by 12 DAI only around 10% of the maximum level of activity present in the megagametophyte was detectable. However, during these same developmental stages, both CAT and ICL steady-state protein, as well as mRNA levels, were still relatively high. To further examine these discrepancies, the relative levels of CAT and ICL *de novo* protein synthesis was measured. Similar to the levels of enzyme activity present in the megagametophyte during these later stages of post-germinative growth, CAT and ICL *de novo* protein synthesis was very low or undetectable for both enzymes. In relation to this, when CAT and ICL were immunoprecipitated from *in vitro* translation products of poly(A)⁺ RNA isolated from the same post-germinative stages, no differences were observed. Apparently, this dramatic decrease in CAT and ICL *de novo* protein synthesis cannot be attributed to a difference in the translatability of their mRNAs during this period. The possibility that there are differences in the translational efficiencies of CAT and ICL mRNAs *in vivo* cannot be ignored. Alternatively, the differential import and assembly of nascent CAT and ICL polypeptides into enzymatically functional tetramers within the glyoxysome could also account for these differences. However, given that an *in vivo* or *in vitro* heterologous glyoxysome import system will have to be developed for loblolly pine to address this possibility, an examination of whether this event is occurring will be very difficult.

At present, the best explanation for the discrepancy between CAT and ICL steady-state protein, *de novo* protein synthesis, and enzyme activities during the later stages of post-germinative growth could be the possibility for the existence of two general classes

of CAT and ICL enzymes; newly synthesized active forms and old inactive forms. Previously, Gershon and Gershon (1970) demonstrated that two populations of ICL were present in nematodes depending on the age of the organism. Using a variety of immunochemical techniques, it was demonstrated that in old animals ICL consists of a proportion of fully active molecules and a larger amount of altered inactive molecules. Gershon and Gershon (1970) concluded that some of the possible mechanisms for alterations that could lead to this inactivation included; (a) modifications of the enzyme after synthesis, (b) changes in the primary structure caused by errors during the synthesis of enzyme molecules, (c) and progressive accumulation of an inhibitor. In later studies, Reiss and Rothstein (1974, 1975) re-examined the mixture of new active and old inactive ICL molecules in nematodes. Reiss and Rothstein (1975) concluded that the lower specific activity of ICL in old organisms was not due to the relative amounts of active versus inactive molecules but two classes of ICL molecules being either fully or only partially inactive. Although only a variation of the conclusions drawn by Gershon and Gershon, Reiss and Rothstein (1975) suggested that this partial inactivation could be attributed to conformational changes of the molecule or an increase affinity to inhibitors. In loblolly pine megagametophytes two populations of ICL and CAT proteins were distinguishable during the later stages of post-germinative growth. On protein blots, the relative levels of steady-state or old ICL and CAT protein remained high. However, and correlated with the dramatic decrease in enzyme activities during this same period, CAT and ICL newly synthesized *de novo* protein levels were very low or undetectable. At present the reason for the dramatic decrease or lack of CAT and ICL protein synthesis (i.e. translation inhibition) remains to be determined. Nevertheless, the possibility does exist that only newly synthesized CAT and ICL proteins are enzymatically active, and the older steady-state pools of each enzyme are inactive due to a conformational change and/or affinity to a substrate or inhibitor.

4.10. Targeting of Loblolly Pine Glyoxysomal Enzymes

Higher plants contain several classes of peroxisomes that differ metabolically and are found during different developmental stages of the plant life cycle (see Olsen and Harada, 1993, 1995 for reviews). Although all contain CAT, the specialized metabolic function of a peroxisome is dictated by the unique set of enzymes that accumulate within each type of organelle. In oilseeds such as loblolly pine, peroxisomes are named glyoxysomes because they possess all of the glyoxylate cycle and β -oxidation enzymes (Breidenbach et al., 1967; Cooper and Beevers, 1969; Hutton and Stumpf, 1969; Ching, 1970; Lopez-Perez et al., 1974). With only a few exceptions (Yamaguchi et al., 1984; Gietl, 1990; Preisig-Müller and Kindl, 1993; Kleff et al., 1994; Kato et al., 1995), plant peroxisomal proteins are encoded by nuclear genes, synthesized on free polyribosomes in the cytosol, and imported into the organelle post-transcriptionally. In a similar fashion CAT, ICL and MS are not detectably modified upon import into the loblolly pine glyoxysome. Loblolly pine CAT, ICL and MS synthesized *in vitro* had the same electrophoretic mobility in SDS-PAGE as their respective counterparts synthesized *in vivo*. Therefore, this implies that the signal for targeting loblolly pine CAT, ICL and MS into the glyoxysome resides on the mature polypeptide.

To date, several types of peroxisomal targeting determinants have been identified, primarily through studies of yeast and mammalian proteins. In total, three distinct signals (i.e. C-terminal, N-terminal, internal signals) have been identified, with a carboxy-terminal SKL motif representing the prototype PTS (see Subramani, 1993 for review). In plants, a survey of the carboxy-terminal sequences of peroxisomal proteins indicated that many, but not all, contain the terminal SKL tripeptide (Olsen and Harada, 1991). In loblolly pine, both PtBs ICL 8 (SRM) and PtBs ICL 12 (ARM) contain a carboxy-terminal tripeptide that is similar to the SKL PTS. Loblolly pine CAT cross-reacted with SKL antiserum. However, it still remains to be determined whether the protein truly contains an SKL PTS, or a variant thereof. Despite one conflicting report (Behari and Baker, 1993), in angiosperms the SKL motif is considered to be both necessary and sufficient to direct the

import of proteins into the peroxisome (Volkita, 1991; Olsen et al., 1993; Trelease et al., 1994). In cotton, Trelease et al. (1994) recently demonstrated the validity of the ICL carboxy terminal tripeptide ARM. This variant of the prototype PTS SKL motif was shown to be both necessary and sufficient for ICL, or a chloroamphenicol passenger protein linked to the ARM tripeptide, import into the peroxisome. At present, it now remains to be determined if the similar SKL PTS motifs, SRM and ARM, located on PtBs ICL 8 and PtBs ICL 12, respectively, function as glyoxysomal targeting signals for ICL in loblolly pine megagametophytes. Given the extensive evolutionary conservation of this SKL targeting signal across plants, mammals, yeast, fungi, trypanosomes (Gould et al., 1990; Keller et al., 1991; Subramani, 1992), as well as gymnosperms, it would seem reasonable to expect that a similar import mechanism likely exists for protein transport into loblolly pine peroxisomes.

4.11. Future Directions

To date, our understanding of the regulatory mechanisms involved in the expression of genes during germination and post-germination of conifer seeds remains limited. As a first step towards understanding these processes in loblolly pine, this thesis has focused on the biochemical and molecular characterization of the developmentally regulated glyoxysomal enzymes CAT, ICL and MS. These enzymes were chosen as markers of study because they represent key hydrolytic enzymes in the biochemical pathways for the mobilization of lipid reserves in oilseeds such as loblolly pine.

As the first step for examining CAT, ICL, and MS gene expression, each enzyme was purified and monospecific antibodies elicited in rabbits. Comparisons of loblolly pine CAT, ICL and MS physicochemical properties and immunological characteristics indicated that these three enzymes were similar to their angiosperm counterparts. However a number of significant differences were also noted. For example, CAT and ICL both display complex mechanisms of protein heterogeneity. Both CAT and ICL consist of subunits with either different molecular masses and/or charge. At present, the molecular basis for this multiplicity remains to be determined.

In general, the most significant contribution of this dissertation to our overall understanding of seed biology is the isolation of cDNA clones for ICL and CAT from loblolly pine. To my knowledge this is the first time enzymes involved in seed lipid reserve mobilization have been cloned from a conifer source. These cDNAs will be important tools for further studies. For example, genomic clones for CAT, ICL and eventually MS will need to be isolated and characterized. Specifically, these clones will allow an examination of the specific interactions between *cis* and *trans*-acting factors required for spatial, temporal, and hormonal regulation of post-germination enzymes such as CAT, ICL, and MS. This later cue, or hormonal regulation of gene expression, appears to involve some form of signaling mechanism between the megagametophyte and the embryo. For example, if the embryo is removed, CAT, ICL, and MS enzyme activity levels in the megagametophyte do not show a dramatic increase following germination (Gifford, unpublished). This same observation has been reported for castor bean CAT (Mullen and Gifford, 1995), and the glyoxysomal enzymes of maize (Skadsen and Scandalios, 1989). Skadsen and Scandalios (1989) suggested that two or more programs of enzyme expression may exist in maize seeds. One program controls the initial tissue-specific induction of a number of specific gene sets, including glyoxysomal protein genes, and another program(s) possibly determine the temporal levels of expression of induced genes. Preliminary data of the expression of glyoxysomal enzymes CAT, ICL and MS in loblolly pine megagametophyte suggests that a similar program, or at least some form of a regulatory signal, is maintained by the presence of the embryo. Now, with the availability of monospecific antibodies and cDNA probes, these tissue-tissue interactions can be examined.

The mechanism of protein targeting to the glyoxysome in loblolly pine megagametophytes appears to be similar to most angiosperms examined to date. Although not a model system, isolated glyoxysomal fractions can be obtained from megagametophytes, thereby permitting an *in vivo* import assay system to be developed. With this system operational, we can begin to examine and compare the mechanisms of protein import into the loblolly pine glyoxysome with peroxisomal import studies currently underway in a number of angiosperm species.

In total, this study, and future molecular approaches to examining the regulation of loblolly pine glyoxysomal gene expression, will eventually lead to the elucidation of the critical developmental mechanism(s) that control them. Consequently, these types of studies will eventually provide the fundamental knowledge of conifer seed biology that can be used by the forest industry to overcome the recalcitrant nature of conifer seed germination.

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